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TITLE: Modulation of Stem Cell Differentiation and Myostatin as an Approach to Counteract Fibrosis in Muscle Dystrophy and Regeneration after Injury

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14. ABSTRACT
In Year 4 the in vivo studies to determine the relative capacity of the wild type muscle derived stem cells (WT MDSC) and their counterparts lacking myostatin obtained from the mouse with genetic inactivation of myostatin (Mst KO MDSC), to repair the notexin-injured gastrocnemius in aged mdx mice, were completed. These aged mice show exacerbated bouts of necrosis and lipofibrotic degeneration that mimic a mild form of Duchenne's muscular dystrophy. Our new results are the first report testing the myogenic capacity of MDSC isolated from transgenic mice with inactivation of either the myostatin or the dystrophin genes, in comparison to the wild type MDSC, both in vitro and in the injured muscle of the aged mdx mice in vivo. Our main new findings are that: a) WT MDSC exert significant myogenic, anti-fat deposition and myofiber repair effects that are evident even in the tissue environment of a severely injured mdx muscle at an age where lipofibrotic degeneration is considerable; and b) these capacities, that were previously shown to be blocked in cell culture conditions, are recovered in Mst KO MDSC when they are implanted in the injured mdx aged muscle setting; however, the implanted Mst KO MDSC do not increase as expected the in vivo regeneration capacity of the MDSC over the one in the myostatin + WT MDSC, presumably because of paracrine effects by myostatin produced by the surrounding tissue. In addition, we have initiated similar studies on the injured diaphragm, the most severely affected muscle in Duchenne's, and new studies on the in vivo pharmacological stimulation of MDSC with nitric oxide donors (molsidomine), and antioxidants (allopurinol, and apocynin), to combat fibrosis and lipofibrotic degeneration, and stimulate MDSC fusion with myofibers.

15. SUBJECT TERMS
Myostatin, muscle dystrophy, stem cells, myogenesis, Oct-4; Duchenne; fibrosis

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Introduction

The **overall objective** of this grant is:

To investigate in the mdx mouse a novel therapeutic approach for Duchenne's muscular dystrophy **(DMD)** based on the inhibition of myostatin **(Mst)** expression and/or activity, for the alleviation of fibrotic and fatty degeneration of the muscle, that would also facilitate the differentiation of transplanted dystrophin+ **(D+)** muscle-derived stem cells **(MDSC)**, in order to ameliorate disease progression.

This will be achieved by: a) comparing the in vitro myogenic and fibrogenic/adipogenic potential of MDSC from D-/Mst+, D+/Mst+ or D+/Mst- mice; b) blocking myostatin expression by gene transfer of myostatin short hairpin RNA (Mst shRNA), or transplantation of D+ MDSC engineered with Mst shRNA, and measuring the myogenic/fibroadipogenic balance, dystrophin expression, and muscle function; and c) combining this with the inhibition of myostatin activity by follistatin.

Description of research accomplishments in Year 4

Some unexpected factors interfered with the completion of some experiments and assays within the original 4-year period. These factors included: a) initial research personnel substitution in the grant at both CDU contract and LABioMed/Harbor-UCLA subcontract, caused by changes in affiliation or line of work for this personnel; and b) technical problems derived from the resistance of the muscle derived stem cells from mdx (mdx MDSC) and myostatin knock out (MstKO MDSC) mice to in vitro myogenesis, the high notexin toxicity for the mdx mice, and the difficult diaphragm interventions, that had to be resolved by prolonging some in vitro and in vivo studies.

For these reasons, in our report for Year 3 we had anticipated a request for a one-year no-cost extension with the following paragraph: *"We plan to complete the schedule below during Year 4, but if needed we will request a no-cost extension with institutional funding support to be able to finish these important experiments".* This request was submitted on February 23, 2011 to the attention of Mr Ayi Ayayi, Contract Specialist, at the USAMRMC-CDMRP, and since we have not received any objection we are assuming that it has been granted. Therefore, we are considering this report as pertaining to Year 4, rather than as a final report that will then be submitted at the completion of the one year no cost extension. The activities in year 4 were:

A. Completion of the first in vivo study and paper integrating in vitro/in vivo results.

The paper listed on the 03/31/10 Year 3 Report as "A-1, to be submitted to the Journal of Endocrinology", was actually sent on 05/12/10 and rejected on 06/08/10 on the basis that it contained a very large amount of data including too much to discuss effectively, and it was only of some endocrine interest. Based particularly on the latter opinion, the scope of the paper was changed to fit it on journals more related to skeletal muscle and stem cells research, rather than endocrinology, by including in vivo experiments, trimming and rearranging the in vitro data, and presenting the integration of the in vitro and in vivo results in a much clearer way. As a result, resubmission was delayed until completing the in vivo study, and the considerably revised paper with deleted and extensive new results, has now been submitted and it is under review **(A-1 rev).** This manuscript includes the following results performed during Year 4

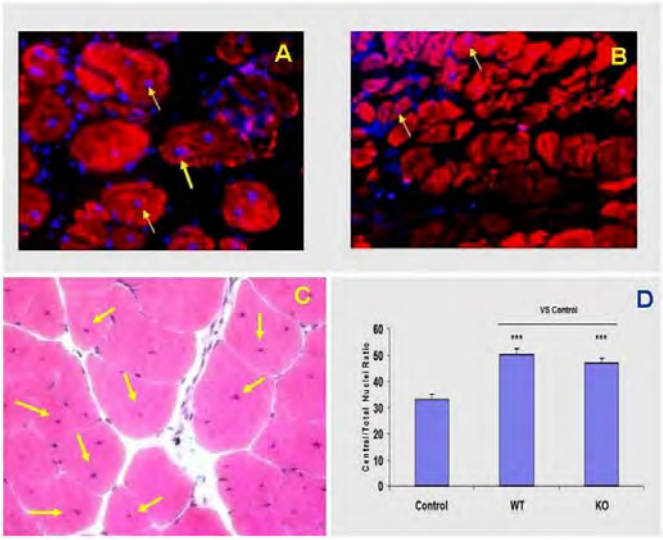

Figure 7. Implanted WT MDSC stimulate myofiber repair in the injured gastocnemius of the old mdx mouse, while Mst KO MDSC act similarly to WT MDSC and mdx MDSC fail to trigger this process. A,B: Aged (10-month old) mdx mice were used to maximize myofiber loss and lipofibrotic degeneration in the gastrocnemius. Muscles wer cryoinjured and implanted with 0.5 x 10^6 DAPI-labeled WT MDSC (A) or mdx MDSC (B), and allowed to undergo repair for 10 days. Frozen muscle sections were stained for MHC-II with Texas red streptavidin, and the merge of blue and red fluorescence was obtained (200X). MDSC nuclei centrally located within myofibers are indicated with yellow arrows. C: Gastrocnemius injury in the aged mdx mice was performed in the two apexes of the muscle with notexin, and muscles were injected 4 days later with saline or with 1.0 x 10^6 WT MDSC or Mst KO MDSC in saline (n=5/group). Repair was allowed to proceed for 3 weeks. Hematoxylin eosin staining was performed in frozen sections and a representative picture shows myofibers from the gastrocnemius implanted with WT MDSC with arrows pointing to abundant central nuclei (200X). D: quantitative image analysis of these tissue sections (WT), in comparison to tissue sections from Mst KO MDSC-implanted mice (KO) and saline-injected controls, based on 12 fields per section, 3 sections per animal. *: $p < 0.05$

WT MDSC stimulate myofiber regeneration and reduce fat infiltration in the injured gastrocnemius of the aged mdx mice, and Mst KO MDSC recover this capacity in vivo. The failure of the mdx MDSC to form myotubes in vitro in any of the previously studied conditions was further supported by a comparison of their relative ability to incorporate nuclei into myofibers in vivo. DAPI-labeled cells were implanted into the cryo-lacerated gastrocnemius of the aged mdx mouse and frozen tissue was examined by MHC II immunofluorescence after 2 weeks. It should be emphasized that the lipofibrotic degeneration in the gastrocnemius of the mdx mice is very mild in young animals and is exacerbated only at an older age (> 8 months of age).

Fig. 7 A (keeping the numbering used in paper A1-rev) shows that the blue fluorescent WT MDSC nuclei are detected in many of the red fluorescent myofibers and many of these nuclei are central, as may be expected from regenerating myofibers (yellow arrows). Other nuclei are seen in the interspersed connective tissue among the fibers. In contrast, the mdx MDSC nuclei remain interstitial, and no intra-myofiber DAPI-labeled nuclei were seen in a series of adjacent sections **(B)**. The Mst KO MDSC were not tested in this model. The WT MDSC significantly stimulated the appearance of central nuclei on hematoxylin/eosin stained frozen tissue sections in the notexin-injured mdx muscle of aged animals in comparison to control injured muscle receiving vehicle **(D).** Surprisingly, the Mst KO

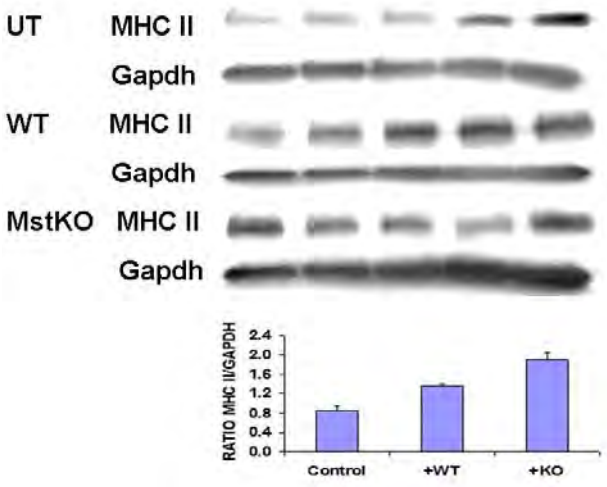

Figure 8. WT and Mst KO MDSC stimulate MHC-II expression in the injured mdx mouse gastrocnemius. Top. Western blot analysis for MHC II and GAPDH (reference gene) in muscle tissue homogenates prepared from the central region of the muscles that were examined histochemically in Figure 7 C. Each lane corresponds to an individual mouse homogenate (n=5/group), and the three gels were run simultaneously. Bottom. Densitometric evaluation of the relative intensity expressed as ratios of the MCH-II and GAPDH bands. *: $p < 0.05$

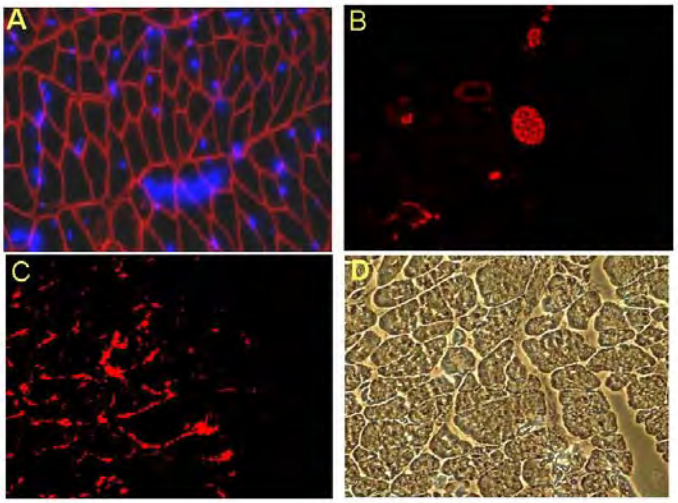

Figure 9. Dystrophin + MDSC restore some dystrophin expression in the injured mdx gastrocnemius, but only in sparse areas. A. Myofibers from the intact gastrocnemius from the WT mouse, the source of WT MDSC, show positive immunofluorescence for dystrophin (nuclei stained with DAPI) (200X). B: WT MDSC implanted in the injured mdx gastrocnemius are still identified after 3 weeks as groups of mononucleated dystrophin+ cells in some tissue sections (200X). C: in other tissue sections, MDSC appear to have fused with the mdx myofibers that show irregular dystrophin + staining; however, most of the sections were negative for dystrophin. D: the same field as in C examined under visible light confirming the integrity of the myofibers including the dystrophin – area.

may have been even higher in the injured tissue.

Both WT and Mst KO skeletal muscles are dystrophin +, as seen on frozen sections by the sarcolemma immunofluorescence around the myofibers **(Fig. 9 A),** and in both cases some residual undifferentiated/non-fused dystrophin + MDSC can be seen 3 weeks after implantation into the dystrophin – injured muscle of aged mdx mice, as groups of interstitial mononucleated MDSC dispersed among some of the fields **(B)**. However, only a few myofibers in the MDSC-implanted mdx muscle show partial dystrophin + staining of the sarcolemma, suggesting that the MDSC myofiber differentiation or fusion is very modest.

MDSC, that failed to undergo myogenesis in vitro, were able to increase significantly the number of central nuclei in the myofibers **(C, D).** However, this stimulation of myofiber repair did not surpass the efficacy of the WT MDSC **(D),** in contrary to what was originally expected from the absence of myostatin in the Mst KO MDSC.

These results were supported by the fact that both WT MDSC and Mst KO MDSC significantly increased the expression of MCH-II in the notexin-injured mdx aged muscle estimated by western blot, as compared to the vehicle-injected muscle, albeit in this case Mst KO MDSC was slightly more effective than WT MDSC **(Fig. 8).** It should be emphasized that this measurement was conducted in the central region of the muscle, distant from the notexin-injured sites at both ends of the muscle used for the tissue section studies, suggesting that the stimulatory effect on MHC-II expression by MDSC

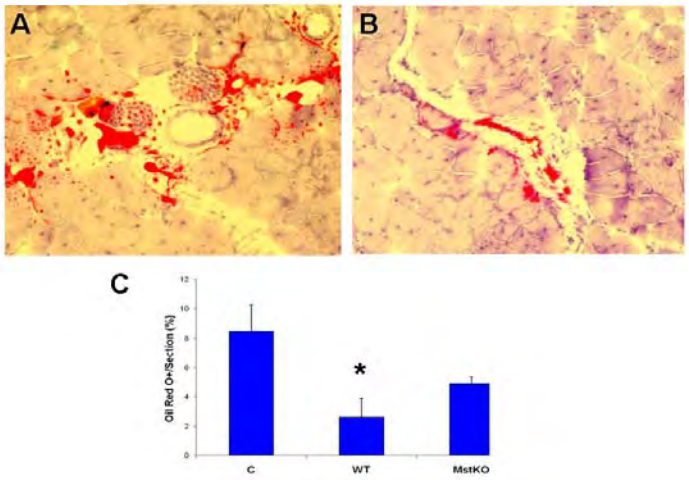

Figure 10. WT MDSC reduce fat deposit in the injured mdx mouse gastrocnemius. A,B: Representative picture of a positive field from frozen tissue sections from the untreated mdx injured gastrocnemius, adjacent to the ones shown on Fig. 8 C, fixed in formalin and stained with Oil Red O, showing mostly interstitial fat and occasional myofiber fat infiltration (200X). Staining in the sections from the muscle implanted with WT and Mst KO MDSC was similar, but in sparser locations. C: quantitative image analysis of the tissue sections from the three rat groups, based on 12 fields per tissue section and the total positive area per section (%), calculated as a mean for 3 adjacent sections per rat, and 5 mdx mice/group. *: $p < 0.05$

As expected, fat infiltration is visible in the injured gastrocnemius from vehicle-injected aged mdx mice, mainly interstitially but also as Oil Red O + small regions around or inside myofibers **(Fig. 10 A, B).** WT MDSC were effective in reducing significantly this fat infiltration, and Mst KO MDSC also induced a decrease, although it was not significant **(C).**

Our new results allowed us to state that paper A1 rev is the first report testing the myogenic capacity of MDSC isolated from transgenic mice with inactivation of either the myostatin or the dystrophin genes, in comparison to the wild type MDSC, both in vitro and in the injured muscle of the aged mdx mice in vivo, that shows a degree of muscle damage and lipofibrotic degeneration more akin to that seen in Duchenne's muscular dystrophy. Our main new findings are listed as d) and e), on the previously shown background of a-c), as described below.

a) in contrast to WT MDSC, Mst KO and mdx MDSC are unable to form myotubes in vitro, or, in the case of mdx MDSC, incorporate their nuclei into the myofibers of injured skeletal muscle in aged mdx mice; this occurs despite no major differences were found among the three MDSC cultures in terms of morphology, replication rates, expression of most members of a subset of key embryonic-like stem cell and other markers, and multi-lineage differentiation other than skeletal myogenic conversion;

b) a fundamental difference is however, that the expression of key genes in myogenesis seen in WT MDSC such as actc1, acta1, and myoD, is virtually obliterated in Mst KO and mdx MDSC;

c) the three types of MDSC are refractory in vitro to modulation or induction of myotube formation by well known regulators of this process or of myofiber number, such as myostatin inhibition or overexpression, follistatin, androgens, nitric oxide, and others, despite some of the respective receptors are expressed in MDSC cultures;

d) WT MDSC exert significant myogenic, anti-fat deposition and myofiber repair effects that are evident even in the tissue environment of a severely injured mdx muscle at an age where lipofibrotic degeneration is considerable; and

e) these capacities, that were previously shown to be blocked in cell culture conditions, are recovered in Mst KO MDSC when they are implanted in the injured mdx aged muscle setting; however, the implanted Mst KO MDSC do not increase as expected the in vivo regeneration capacity of the MDSC over the one in the myostatin + WT MDSC, presumably because of paracrine effects by myostatin produced by the surrounding tissue.

B. Initiation of second and third vivo studies In addition, we started the following activities that had been planned in the Year 3 report for Year 4:

1. Additional mdx mice were added to the animals already performed during Year 3, to reach an n=5'group for the parallel study on the diaphragm repair after notexin injury. This is the critical muscle affected in Duchenne's dystrophy (main cause of lethality) and in the mdx mouse, where lipofibrotic degeneration is also exacerbated in most muscles at ages >8-10 months. Also, another group of 5 more animals was added for treatment with Mst KO MDSC. This completes three groups of notexin-injured aged muscle gastrocnemius: a) WT MDSC; b) Mst KO MDSC; c) vehicle
2. The diaphragm frozen tissue sections are currently being studied for Oil Red O for fat deposition and Sirius Red and ASMA immunostaining for fibrosis. These are the initial assays scheduled to conclude whether MDSC can correct the severe dystrophy both in the aged gastrocnemius and in the aged diaphragm, and myostatin inactivation improves this process. This will be the subject of a separate paper.
3. Another study with 5 animal groups (N=6/group; total: 30 mice) has been started with all the planned outcomes and procedures as in the previous in vivo studies, including ASMA western blot for fibrosis, but implanting only WT MDSC into only the gastrocnemius of the

aged mdx mouse, under the following simultaneous 3 weeks continuous pharmacological treatments acting as adjuvant of MDSC proliferation, differentiation, and terrain conditioning:

a. Long-term stability nitric oxide donor (molsidomine, ip), assumed to have antifibrotic effects by producing both nitric oxide and cGMP, as shown by us, and that also stimulates satellite cell fusion during muscle repair. Two groups: molsidomine alone; molsidomine and WT MDSC

b. Antioxidant agents, based on the combination of oral apocynin as NADPH oxidase inhibitor and oral allopurinol as xanthine oxidoreductase inhibitor, to counteract oxidative stress as a profibrotic effector, as previously shown by us**.** Oxidative stress is a major factor in the skeletal muscles of Duchenne patients and the mdx mice. Two groups: allopurinol + apocynin alone; both drugs with WT MDSC

c. Control animals treated with vehicle. One group

Finally, the parallel studies, where we applied WT MDSC alone and concurrently with a continuous long-term administration of a PDE 5 inhibitor to elevate in a sustained way the cGMP levels in the heart, in order to counteract fibrosis and stimulate cardiomyocyte regeneration in a rat model of myocardial infarction, have been re-submitted for publication **(A-2 rev)**. As already stated, this study has supported a pharmacological antifibrotic approach (molsidomine as nitric oxide donor) that we intend to explore in the no cost extension as an adjuvant to the effects of the WT MDSC implanted into the skeletal muscle of the mdx mouse. Another paper, where we demonstrated that molsidomine or allopurinol act as antifibrotic or antioxidant agents in the context of smooth muscle fbrosis, has been published **(B-1).**

The more pertinent essential details are presented in the abstract for paper A-1 rev below. The full text is included in the appendix.

A-1 rev. Tsao J, Vernet D, Gelfand R, Kovanecz I, Nolazco G, Gonzalez-Cadavid NF.myostatin genetic inactivation inhibits myogenesis by muscle derived stem cells in vitro but not when implanted in the mdx mouse muscle. 03/11, submitted

Stimulating the commitment of implanted dystrophin + muscle derived stem cells **(MDSC)** into myogenic as opposed to lipofibrogenic lineages is a promising therapeutic strategy for Duchenne muscular dystrophy **(DMD)**. To examine whether counteracting myostatin, a negative regulator of muscle mass and a pro-lipofibrotic factor, would help this process, we compared the in vitro myogenic and fibrogenic capacity of MDSC from wild type **(WT)**, myostatin knockout (**Mst KO**), and mdx (DMD model) **(mdx)** young mice under various modulators, the expression of key stem cell and myogenic genes, and the capacity of these MDSC to repair the injured gastrocnemius in aged mdx mice with exacerbated lipofibrosis. Surprisingly, the potent in vitro myotube formation by WT MDSC was refractory to modulators of myostatin expression or activity, and the Mst KO and mdx MDSC failed to form myotubes under any condition, despite all MDSC expressed Oct-4 and various stem cell genes and differentiated into other lineages. The genetic inactivation of myostatin or dystrophin in MDSC was associated with silencing of critical genes for early myogenesis (Actc1, Acta1, and MyoD). WT MDSC implanted into the injured mdx gastrocnemius improved myofiber repair and reduced fat deposition. In contrast to their in vitro behavior, Mst KO MDSC acted similarly to WT MDSC in vivo. In conclusion, myostatin gene inactivation in MDSC silences key genes for myotube formation, which are similar to the ones down-regulated in mdx MDSC, and the restored myogenesis in the implanted Mst KO MDSC may be elicited by a putative reactivation of these genes by the injured muscle.

Bulleted list of key research accomplishments in Year 4

- The studies in vivo on the comparative ability of MDSC from the WT and Mst KO mice to stimulate the repair of the injured gastrocnemius in the aged mdx mouse, a model for Duchenne's muscular dystrophy, were completed and integrated in manuscript **A-2 rev**, showing that implantation of WT MDSC into the muscle improved myofiber regeneration and reduced fat deposit in gastrocnemius, and that the Mst KO MDSC lacking myostatin were nearly as efficient in these processes.
- Our findings suggest the important concept that that the restoration of the myogenic ability of Mst KO MDSC (that was completely inhibited in vitro as shown in our initial experiments) is due to the paracrine influence of the injured muscle environment that may trigger the activation of genes that are key regulators of myogenesis, specifically spp1, actc1, acta1, notch 2, and myoD, that are neither expressed in Mst KO mDSC nor in mdx MDSC.
- In turn, the results proved that the lack of myostatin in the implanted Mst KO MDSC is not sufficient to increase the in vivo regeneration capacity of stem cells over the one in WT MDSC with normal myostatin expression, presumably because of putative paracrine effects by myostatin produced by the surrounding tissue. This rules out the initial hypothesis that engineering MDSC with shMst RNA would improve repair even in the myostatin + milieu of the host mdx muscle.
- Finally, the demonstration in pallalel studies in alternative systems **(A-2 rev, A-3)** of the antifibrotic effects of nitric oxide donors and cGMP stabilizers, like molsidomine and PDE5 inhibitors, and of antioxidants, like allopurinol, supports our ongoing experimental design to directly investigate whether they stimulate the repair ability of WT MDSC on the injured muscle of aged mdx mice, in lieu of the initial goal of inhibiting myostatin.

Reportable outcomes (Year 4)

A. Papers acknowledging this grant (see Appendix)

Resubmissions:

A-1 rev. Tsao J, Vernet D, Gelfand R, Kovanecz I, Nolazco G, Gonzalez-Cadavid NF (2011) Myostatin genetic inactivation inhibits myogenesis by muscle derived stem cells in vitro but not when implanted in the mdx mouse muscle, submitted.

A-2 rev. Wang J S-C, Nolazco G, Kovanecz I, Vernet D, Kopchok GE, Chow S, Keyhani A, White RA, Gonzalez-Cadavid NF (2011) Effects of muscle derived stem cells and long-term treatment with a PDE5 inhibitor on myocardial infarction in a rat model. PLOS One, submitted

B. Related papers that support part of the no cost extension experiments

B-1. Ferrini MG, Moon J, Rivera S, Rajfer J, Gonzalez-Cadavid NF (2011) Amelioration of diabetes-induced fibrosis by antioxidant and anti-TGFβ1 therapies in the penile corpora cavernosa in the absence of iNOS expression. BJU Int, in press

C. Abstracts and presentations related to results in the current grant

C-1. Chow SL CA, Kovancz I, Wang JSC, Vernet D, Kopchock G, White RA, Gonzalez-Cadavid NF (2010). Inflammatory Biomarkers in Left Ventricular Remodeling under Stem Cell

and Pharmacological Treatment in a Rat Model of Myocardial Infarction. Heart Failure Society of America (HFSA); September 14, 2010; San Diego, CA2010. p. S33.

C-2. Wang JS KI, Vernat D, Nolazco G, Kopchock G, Chow SL, White RA, Gonzalez-Cadavid N. . Effects of long-term continuous treatment with sildenafil alone or combined with muscle derived stem cells (MDSC) on myocardial infarction in a rat model. American Heart Association BCVS and American Heart Association Scientific Sessions; July, 19, 2010 and November 15, 2010; Rancho Mirage, CA and Chicago, IL.

D. Related grants, funded

The following grant application, which uses results obtained during Year 4 of this grant, was awarded:

D1. Gonzalez-Cadavid NF (PI, pilot grant) U54-RR026138-02 (Norris K, Program Director) Therapy of diabetes-related critical limb ischemia with muscle derived stem cells and NO donors. 03/01/11-02/28/12

E. Unrelated grants, funded

The following grant applications were awarded:

E1. Gonzalez-Cadavid NF (PI) NIH R21ES019465-01 Bisphenol A effects on the peripheral mechanisms of penile erection. 09/01/10-08/31/12

E2. Gonzalez-Cadavid (PI, pilot grant) U54 CA14393-01 (Vadgama J, Program Director) Potential oncogenic effects of alcohol on breast stem cells 01/01/10-08/31/12

F. Unrelated grants, submitted

F1. Gonzalez-Cadavid NF (PI) NIH NIEHS 1U01ES020887-01 Cellular-molecular signature and mechanism of BPA effects on penile erection. 10/01/11-9/30/15

F2. Nicholas S/Gonzalez-Cadavid NF (Co-PIs) NIH NIDDK R21 Effects of diabetes on stem cell cross talk in renal tissue repair. 07/01/11-06/30/13.

F3. Gonzalez-Cadavid NF (PI) Animal Models of Diabetes Complications Consortium. A diabetes mouse model for studying endogenous/exogenous stem cell interaction. 10/01/10-09/30/11. Not funded

Conclusions

Program for the no cost extension

- As stated in the request for the one year no cost extension, institutional funds will cover Dr James Tsao to work for this project at 30% effort until completion. Additional expenses for animal and supplies costs will also be covered from institutional funds. Dr. Gonzalez-Cadavid will donate a 2% effort during the no-cost extension to supervise Dr. Tsao and write the additional papers and the final report for the grant. Should there be an unobligated balance it will be used exclusively at CDU to support salary and fringe benefits for James Tsao.
- It is estimated that the work described below will take about 9 months, and then 2-3 more months are needed to analyze the data, prepare the figures, write and submit both an additional paper and the final report.
- The specific objectives to be fulfilled during the no-cost extension are:
    a. Finalize the second paper pertaining to the study of the effects of WT and Mst KO MDSC on the repair of the notexin-injured diaphragm in the aged mdx mice, by defining

lipofibrotic degeneration (Oil Red O, Sirius Red), myofiber repair (central nuclei in hematoxylin/eosin, MHC II, dystrophin), and if possible oxidative stress (xanthine oxido-reductase, NADPH oxidase)

b. Finalize the parallel ongoing study on the effects of molsidomine and the combination of allopurinol and apocynin in stimulating the repair capacity of MDSC on the injured gastrocnemius muscle in aged mdx mice, by measuring the same outcomes as in a, including ASMA for myofibroblast formation. If new funding becomes available, an additional group will be studied: follistatin, that at present is too expensive to pursue because of the cost of follistatin. Myostatin antibodies are also too expensive, and the Mst shRNA effects on the host mdx muscle may act only in very restricted regions contrary to what was originally envisaged, plus the fact that the genetic inactivation in the Mst KO MDSC did not achieve the expected stimulation, so these approaches will not be pursued.
c. The most effective group in reducing lipofibrotic degeneration and stimulating myofiber repair in b, as well as a control with MDSC alone, will then be subjected to the functional studies originally planned.
d. It is doubtful that the limited funding available will allow to continue with the in vitro studies reported for Year 3 that we proposed to continue in Year 4 and could not be conducted for the reasons explained above. However, these are listed just in case additional funding is obtained:

   d1. try to mimic in vitro the paracrine and juxtacrine environment of the MDSC implanted in the muscle, such as with C2C12 myoblasts in dual cultures under different conditions including pharmacological modulation, to determine whether the balance of myogenic/lipofibrogenic differentiation can be improved by these interactions;

   d2. exploring the mechanism of the Mst KO MDSC inhibition of myogenesis by WT MDSC in dual juxtacrine cultures, to find out which are the genes down-regulated by this interaction in the WT MDSC;

   d3.conducting gain-of-function experiments for some of the genes that we found to be considerably down-regulated by myostatin or dystrophin genetic inactivation in the Mst KO and mdx MDSC, to try to find a way to activate myogenesis in the endogenous MDSC of the mdx mouse

MYOSTATIN GENETIC INACTIVATION INHIBITS MYOGENESIS BY MUSCLE DERIVED STEM CELLS IN VITRO BUT NOT WHEN IMPLANTED IN THE mdx MOUSE MUSCLE

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Running Title: Myostatin or dystrophin silencing and muscle stem cells

Abbreviations: AdV-CMV-Mst375: adenovirus construct expressing the mouse myostatin full-length cDNA under the CMV promoter**; AdV-Mst shRNA:** shRNA against myostatin RNA; **ASMA:** α-smooth muscle actin; **MDSC**: muscle derived stem cells; **Mst KO**: myostatin knock out mouse; **QIA:** quantitative image analysis**; TGF β1**: transforming growth factor β1. **VSEL:** very small embryonic-like stem cells. **WT:** wild type mouse.

Submitted: March 28, 2010

ABSTRACT

Stimulating the commitment of implanted dystrophin + muscle derived stem cells **(MDSC)** into myogenic as opposed to lipofibrogenic lineages is a promising therapeutic strategy for Duchenne muscular dystrophy **(DMD)**. To examine whether counteracting myostatin, a negative regulator of muscle mass and a pro-lipofibrotic factor, would help this process, we compared the in vitro myogenic and fibrogenic capacity of MDSC from wild type **(WT)**, myostatin knockout (**Mst KO**), and mdx (DMD model) **(mdx)** young mice under various modulators, the expression of key stem cell and myogenic genes, and the capacity of these MDSC to repair the injured gastrocnemius in aged mdx mice with exacerbated lipofibrosis. Surprisingly, the potent in vitro myotube formation by WT MDSC was refractory to modulators of myostatin expression or activity, and the Mst KO and mdx MDSC failed to form myotubes under any condition, despite all MDSC expressed Oct-4 and various stem cell genes and differentiated into other lineages. The genetic inactivation of myostatin or dystrophin in MDSC was associated with silencing of critical genes for early myogenesis (Actc1, Acta1, and MyoD). WT MDSC implanted into the injured mdx gastrocnemius improved myofiber repair and reduced fat deposition. In contrast to their in vitro behavior, Mst KO MDSC acted similarly to WT MDSC in vivo. In conclusion, myostatin gene inactivation in MDSC silences key genes for myotube formation, which are similar to the ones down-regulated in mdx MDSC, and the restored myogenesis in the implanted Mst KO MDSC may be elicited by a putative reactivation of these genes by the injured muscle.

Key words: dystrophin, mdx mouse, Duchenne, fibrosis, dystrophy

INTRODUCTION

The lipo-fibrotic degeneration of skeletal muscle, i.e. excessive deposition of endomysial collagen, other extracellular matrix, and fat, characterizes muscle dystrophy, and in particular Duchenne muscular dystrophy **(DMD) (1,2),** as seen also in its animal model, the mdx mouse **(3-5).** This process, associated with inflammation and oxidative stress **(6),** is partially responsible for the severe muscle contractile dysfunction in DMD and the mdx mouse, mainly caused by the bouts of myofiber necrosis caused by dystrophin genetic inactivation, that in the gastrocnemius are rather mild in young animals but become particularly severe after 8-10 months of age **(4).** Dystrophic muscle fibrosis is not only a major factor for DMD mortality**,** but also hampers the uptake and survival of cells implanted for potential therapeutic approaches **(7),** and/or may drive their differentiation into myofibroblasts **(4).** Therefore, trying to ameliorate this process while stimulating myogenesis constitutes an ancillary strategy to favor repair and regeneration of dystrophic muscle tissue, even under ineffective or absent dystrophin replacement.

Although pharmacological approaches to combat muscle lipofibrotic degeneration and the underlying chronic inflammation are being widely investigated, biological factors such as myostatin, the main negative regulator of muscle mass **(8),** are also potential key targets. Myostatin, a member of the TGFβ family, aggravates muscle dystrophy not only as an anti-myogenic agent but also as a pro-fibrotic and adipogenic factor **(9-14)**. Inhibition of myostatin by using its propeptide, shRNA, or specific antibodies, improves myogenesis and reduces fibrosis in the mdx mouse. The same effects are generated in response to genetic deletion of myostatin in the myostatin knock-out **(Mst KO)** mouse, where myofiber hypertrophy is associated with less fat and reduced fibrosis **(15-20).** The effects of myostatin on myofiber size and on myogenesis in vitro are counteracted by testosterone in part by reducing myostatin levels **(21-23)**, and

indirectly through nitric oxide stimulating myoblast replication, differentiation, and fusion into myotubes **(24,25)**.

It is assumed that in the dystrophic or injured muscle, tissue repair and the opposite process of lipofibrotic degeneration involve not only the differentiation of progenitor satellite cells and fibroblasts into myofibers and myofibroblasts, respectively, but also the modulation of lineage commitment by stem cells present in the adult muscle **(26-28)**. These stem cells have been isolated from the rodent and human skeletal muscle and named in general as muscle-derived stem cells **(MDSC)**, because they have the ability to differentiate in vitro into multiple cell lines, and to generate myofibers, osteoblasts, or smooth muscle cells after implantation into the skeletal muscle, bone, or corpora cavernosa and vagina, respectively **(26-30)**. They are not satellite cells and may act also by secreting paracrine growth factors that are believed to modulate the differentiation of endogenous stem cells or the survival of differentiated cells in the tissue. However, the roles of MDSC in the biology and pathophysiology of the skeletal muscle are largely unknown.

Myostatin modulates the differentiation of pluripotent cells in vitro, albeit in some cases with conflicting outcomes **(14,32-34)**. It also inhibits the proliferation and early differentiation of both satellite cells from the skeletal muscle and of cultured myoblasts, and improves the success of their in vivo transplantation **(35-37)**. To our knowledge, no reports are available on myostatin effects on MDSC differentiation, either in vitro or in the context of repairing the exacerbated lipofibrosis in the injured muscle of aged mdx mice.

MDSC obtained from wild type **(WT)** mice have been tested experimentally aiming to trigger repair of the mdx muscle with variable results **(38-42)**, but they appear to be superior in this respect to myoblasts or satellite cells **(43).** However, some of the main limitations of myoblast therapy when translated from the murine models into DMD and other human muscle dystrophies may also affect the MDSC and other types of stem cells **(44).** Therefore, it is a therapeutic goal to enhance the repair capacity of WT MDSC by in vitro or in vivo modulation of

their multilineage potential, and to stimulate or even awake endogenous stem cells of dystrophic muscle to regenerate myofibers while avoiding differentiation into cells responsible for lipofibrotic degeneration. However, no reports are available on this potential in vitro modulation of MDSC, or the effects that myostatin or dystrophin gene inactivation exert on this balance.

In the current study we have investigated the in vitro myogenic versus fibrogenic and adipogenic differentiation of MDSC isolated from the mdx and Mst KO skeletal muscle vis-à-vis the WT counterpart, and the potential manipulation of these processes by modulating myostatin expression or activity, and by other putative regulators of muscle mass and fibrosis. Their differential in vitro features in terms of the expression of some key stem cell and myogenic genes, and the repair ability of Mst KO MDSC in the injured mdx muscle, were also studied. The ultimate goal is to gain a preliminary insight on how in vitro preconditioning of MDSC by pharmacological or gain of function approaches may modulate their capacity to repair dystrophic skeletal muscle, to design in vivo pharmacological interventions that may mimic these processes, and even myostatin blockade in the host muscle to activate myogenesis in the endogenous dystrophin negative MDSC. .

METHODS

Animals

Only male mice were used throughout, and unless stated they were 12-15 weeks old mdx mice (C57BL/6/10ScSn-Dmd^{mdx}), referred to here as “mdx”, obtained from Jackson Laboratories (Bar Harbor, ME). At this age the first round of muscle necrosis and regeneration has already subsided (“stable phase”). In the in vivo experiments mice were allowed to reach 10 months of age, when lipofibrotic degeneration is most evident, not only in the diaphragm but in the gastrocnemius**.** Mst knock-out mice (C57BL/6J/Mst-/-), referred to here as “Mst KO”, are regularly maintained and bred in our vivarium on a BL/6 background **(45)**, derived from the

original strain on a BalBc background**.** Aged-matched wild type control mice (C57BL/6J), referred to here as "WT", were also from Jackson Laboratories. The Oct-4 Pr-gfp transgenic mouse **(46)**, referred to here as Oct-4 and also bred in our vivarium, expresses the green fluorescent protein Egfp under the control of the gene enhancers and promoter of the embryonic stem cell gene Oct-4.

MDSC isolation from different strains of mice

Hind limb muscles from the WT, Mst KO, and mdx male mice (12-16 weeks old) were subjected to the preplating procedure to isolate MDSC **(5,29,30,43)**. Tissues were dissociated using sequentially collagenase XI, dispase II and trypsin, and after filtration through 60 nylon mesh and pelleting, the cells were suspended in GM-20 (Dulbecco's Modified Eagle's Medium (DMEM) with 20% fetal bovine serum (FBS). Cells were plated onto collagen I-coated flasks for 1 hr (preplate 1 or pP1), and 2 hrs (preplate 2 for pP2), followed by sequential daily transfers of non-adherent cells and re-platings for 2 to 6 days, until preplate 6 **(pP6).** The latter is the cell population containing MDSC. Cells were maintained in GM-20 on regular culture flasks (no coating) and used in the 14th-28th passage. WT MDSC have been maintained in our laboratory for at least 40 generations with the same, or even increasing, growth rate**.** In the case of the Oct-4 Pr gfp MDSC ("Oct-4 MDSC") the same procedure was applied. Green fluorescent single cells or clusters/spheroids were monitored, as well as their morphological features (large nucleus, easily detachable, <10 um)**.** In some cases, Sca1+ cells were selected with immunobeads (Milteny) coated with antibody against the selected antigen (Sca1) **(29)**.

Stem cell characterization, differentiation, and modulation

MDSC cultures from the three mouse strains were analyzed for the expression of stem cell markers below, on collagen-coated 6-well plates and 8-removable chamber plates. Multipotency was analyzed in 2-week incubations with GM-20 or GM-10 (GM with 10% fetal

bovine serum) supplemented or not with 10 nM DMSO or 5 ng/ml TGFβ1, or, to induce myofiber formation, after reaching confluence, for 2-3 weeks with GM-10 (Hedrick's medium) supplemented with 5% horse serum and 50 µm hydrocortisone to promote proliferation, a key event in myogenic differentiation) **(47),** or as described. In certain cases, cultures were treated with or without 20 µM 5'-azacytidine (AZCT) in GM-20 for 3 days to induce multipotency, prior to switching them to the appropriate medium **(11,14,47).**

For the tests on the modulation of MDSC skeletal myotube formation by various factors, cells were allowed to reach confluence, switched to Hedrick's medium, and incubated for 2 weeks with 2 µg/ml recombinant 113 amino acid myostatin protein (R-Mst), a recombinant 16 kDa protein containing 113 amino acid residues of the human myostatin protein (BioVendor Laboratory Medicine Inc., Palackeho, Czech Republic) **(14)**, or with a recombinant mouse follistatin protein (RD Systems, Minneapolis, MN) at 0.2 ug /ml **(11,14),** changing medium twice a week. In other experiments**,** incubations with the monoclonal (Chemicon International, Temecula, CA) and polyclonal (Millipore Corp, Billerica, MA) antibodies against myostatin (1/20) were substituted for the previous treatments Alternatively, the adenoviruses expressing the mouse myostatin full-length cDNA under the CMV promoter **(AdV-CMV-Mst375)** and an shRNA, which targets myostatin RNA and inhibits more than 95% of myostatin gene expression **(11,14,17) (AdV-Mst shRNA)** were transduced into MDSC at 80% confluence. Then cells were switched to Hedrick's medium as above. For a potential hormonal regulation of MDSC differentiation, confluent MDSC in Hedrick's medium were incubated with testosterone or dihydrotestosterone (Sigma Aldrich, St Louis, Mo) at 100 and 20 nM, respectively **(17)**, or thyroid hormone (T3) (Sigma Aldrich) at 2.4 ng/ml as above. Finally, SNAP (Alexis Biochemicals, San Diego, CA) was used a as a nitric oxide donor at 50 um on confluent MDSC

Implantation of MDSC into skeletal muscle

Mice were treated according to National Institutes of Health (NIH) regulations with an Institutional Animal Care and Use Committee-approved protocol. In one experiment, the WT and mdx MDSCs (0.5–1.0 × 10^6 cells/50 µL saline) were labeled with the nuclear fluorescent stain 4',6-diamidino-2-phenylindole (DAPI) **(29,30),** and implanted aseptically under anesthesia into the surgically exposed tibialis anterior of 10 month old mdx mice. The muscle had been cryoinjured by pinching it for 10 seconds with a forceps cooled in liquid N2 immediately prior to implantation. Control mice with muscle cryoinjury received saline. Mice were euthanized, the tibialis excised and subjected to cryoprotection in 30% sucrose, embedding in OCT and cryosectioned.

In another experiment, the DAPI-labeled WT and Mst KO MDSCs (0.5 × 10^6 cells/50 µL GM) were implanted into the central region of the surgically exposed left gastrocnemius of 10 month old mdx mice, which four days earlier had been injured with two injections of notexin in both tips of the muscle (total: 0.2 ug in 10 ul saline), using MDSC-untreated muscle injured mice as controls that were injected with saline (n=5/group). Mice were euthanized at 3 weeks, the gastrocnemius excised and a section around the site of notexin injection was used for cryosectioning. The remainder tissue was kept frozen at -80C.

Immunocytochemistry dual immunofluorescence

Cells on collagen-coated eight-well removable chambers, fixed in 2% p-formaldehyde, and 10 um unfixed frozen tissue sections, were reacted **(10,11,14,17,29,30,45)** with some of the following primary antibodies against: (1) human myosin heavy chain fast, detecting both MHC-IIa and MHC-IIb); monoclonal, 1:200 Vector Laboratories, Burlingame, CA, USA), a marker for skeletal myotubes and myofibers; (2) human αSMA (mouse monoclonal in Sigma kit, 1:2, Sigma Chemical, St Louis, MO, USA), a marker for both SMC and myofibroblasts; (3) neurofilament 70 (NF70; mouse monoclonal, 1:10, Millipore, Billerica, Massachusetts, USA); (4) Dystrophin (rabbit polyclonal, 1:200 Abcam, Cambridge, Massachusetts, USA); (5) Sca-1 (mouse

monoclonal, 1:100, BD Pharmingen, San Jose, CA) and M.O.M blocking kit (Vector, Burlingame, CA), and 6) Oct-4 (rabbit polyclonal, 1:500, BioVision, Mountain View, CA). When MDSC DAPI on 8-well chambers were not previously tagged with DAPI, all nuclei were stained with coverslips with DAPI anti-fading emulsion

Cultures or tissue sections not involving DAPI labeling were subjected to immuno-histochemical detection by quenching in 0.3% H_2O_2, blocking with goat (or corresponding serum), and incubated overnight at 4 °C with the primary antibody. This was followed by biotinylated anti-mouse IgG (Vector Laboratories), respectively, for 30 min, the ABC complex containing avidin-linked horseradish peroxidase (1:100; Vector Laboratories), 3,3' diaminobenzidine, and counterstaining with hematoxylin, or no counterstaining. For cells labeled with DAPI, fluorescent detection techniques were used. The secondary anti-mouse IgG antibody was biotinylated (goat, 1:200, Vector Laboratories) and this complex was detected with streptavidin-Texas Red. After washing with PBS, the sections were mounted with Prolong antifade (Molecular Probes, Carlsbad, CA, USA). Negative controls in all cases omitted the first antibodies or were replaced by IgG isotype. In the case of Oct-4, streptavidin-FITC was used (green fluorescence).

In tissue cryosections for experiments involving DAPI-labeled cells (10 um), tissue sections were processed in regions where the DAPI + cells could be detected. Muscle fibers were either stained with hematoxylin/eosin, or by MHC-II antibody, either by Texas red fluorescence as above, or with the diaminobenzidine tetrahydrochloride-based detection method (Vectastain-Elite ABC kit; Vector Labs), counterstaining with Harris hematoxylin. Tissue sections that were incubated with mouse IgG instead of the primary antibody served as negative controls. The sections were viewed under an Olympus BH2 fluorescent microscope, and cell cultures under an inverted microscope. In some cases, the cytochemical staining was quantitated by image analysis using ImagePro-Plus 5.1 software (Media Cybernetics, Silver Spring, MD, USA) coupled to a Leica digital microscope bright field light fluorescence microscope/VCC video

camera. After images were calibrated for background lighting, integrated optical density (IOD=area x average intensity) was calculated.

Gene expression profiles

Pools of total cellular RNA from three T25 flasks for each MDSC that were incubated with DMEM supplemented with FBS at 20% were isolated with Trizol-Reagent (Invitrogen, Carlsbad, CA), and subjected to DNAse treatment, assessing RNA quality by agarose gel electrophoresis. cDNA gene microarrays (SuperArray BioScience Corp., Frederick, MD) **(11,29)** were applied, using the mouse stem cell (OMM-405), Oligo GEArray microarray: Biotin-labeled cDNA probes were synthesized from total RNA, denatured, and hybridized overnight at 60˚C in GEHybridization solution to these membranes. Chemiluminescent analysis was performed per the manufacturer's instructions. Raw data were analyzed using GEArray Expression Analysis Suite (SuperArray BioScience Corp., Frederick, MD). Expression values for each gene based on spot intensity were subjected to background correction and normalization with housekeeping genes, and then fold changes in relative gene expression were calculated

The expression of some of the down- or up-regulated genes detected above was examined on 1 ug RNA isolated from consecutive similar incubations performed in triplicate by reverse transcription (RT) using a 16-mer oligo(dT) primer, as previously described **(11,29),** and the resulting cDNA was amplified using PCR in a total volume of 20 µl. The locations of the primers utilized for the quantitative estimation of mouse myostatin mRNA were nt 136–156 (forward) and 648–667 (reverse), numbering from the translation initiation codon (later called F2/R2) as previously described. For mouse GAPDH primers, sequences were from the mRNA sequence NM_008084.2, using a forward primer spanning nts 778-797 and reverse primer spanning nts 875-852, with a product length of 98 nt.

Additional primers were designed using the NCBI Primer Blast program applied to mRNA sequences and synthesized by Sigma-Aldrich. Numbering refers to the length in NT from

the 5' end of the mRNA: Acta1 (skeletal muscle actin) NM_009606.2 (forward 501-520 and reverse 841-822, product length 341); Actc1 (cardiac actin) NM_009608.3 (forward 38-58 and reverse 554-530, product length 517); 4) MyoD NM_010866.2 (forward 515-534 and reverse 1013-994, product length 499); and 5) Pax3 NM_008781.4 (forward 1164-1183 and reverse 1893-1874, product length 730). The number of PCR cycles used for each primer set are stated in Fig. 6. All primers were designed to include an exon-exon junction in the forward primer except for Gapdh and MyoD1. negative controls omitted the reverse transcriptase.

Protein expression by western blots

Cells were homogenized in boiling lysis buffer (1% SDS, 1mM sodium orthovanadate, 10 mM Tris pH 7.4 and protease inhibitors, followed by centrifugation at 16,000 *g* for 5 min **(10,11,14,17,29,30,45).** 40 µg of protein were run on 7.5% or 10% polyacrylamide gels, and submitted to transfer and immunodetection with antibodies against: 1) human αSMA (monoclonal, 1/1000, Calbiochem, La Jolla, CA); 2) Oct-4, as for immunohistochemistry; 3) MyoD (rabbit polyclonal 1/200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA); 4) MHC (fast), as for immunohistochemistry; 5) TGF-β1 (rabbit polyclonal 1:1000; Promega Corporation); 6) myostatin (rabbit polyclonal 1:1000; Chemicon International Inc, Temecula, CA), 7) ActRIIb (monoclonal, 1/1000, Abcam, Cambridge, MA); and 8) GAPDH (mouse monoclonal, 1/3000, Chemicon). Membranes were incubated with secondary polyclonal horse anti-mouse or anti-rabbit IgG linked to horseradish peroxidase (1:2000; BD Transduction Laboratories, Franklin Lakes, NJ, or 1:5000, Amersham GE, Pittsburgh, PA,) and bands were visualized with luminol (SuperSignal West Pico, Chemiluminescent, Pierce, Rockford, IL). For the negative controls the primary antibody was omitted.

Statistics

Values are expressed as the mean (SEM). The normality distribution of the data was established using the Wilk–Shapiro test. Multiple comparisons were analyzed by a single factor ANOVA, followed by *post hoc* comparisons with the Newman–Keuls test. Differences among groups were considered statistically significant at $P < 0.05$.

RESULTS

MDSC cultures from the Mst KO and mdx mice resemble their counterparts from WT mice in morphology, replication, cell markers and multipotent differentiation

WT MDSC (pP6 fraction) formed in vitro the most robust skeletal myotubes (see next section) at about passage 13, and the three types of MDSC were compared from passages 10-28. The morphology of the proliferating three cultures was similar, and the replication times for the WT and mdx MDSC were also similar (21.2 vs 19.8 hs, respectively), with the Mst KO MDSC replicating slowe (27.0 hs). This morphology and replication pattern continued throughout the 13-28 passages period of study.

The WT MDSC culture was previously shown to be Sca 1+ **(30),** and now the key embryonic stem cell marker, Oct-4, was also found to be expressed in a large proportion of the three MDSC cultures growing in DM-20 in sub-confluent cultures. It is mainly localized in the nuclei, where the transcriptionally active isoform, the Oct-4A, is expected to occur **(Fig. 2 A).** There is some additional cytoplasmic staining denoting the stem cell-unrelated Oct-4B variant **(48).** This is confirmed by western blot **(B, left),** showing an equal and considerable expression of the 45 kDa Oct-4A stem cell-active protein among the three lines, and a lower content of the stem cell-inactive 33 kDa Oct-4B. That MDSC have some embryonic stem cell features is also suggested from two additional findings. First, MDSC were obtained from a the Oct-4 Pr-gfp transgenic mouse and shown to express gfp (green fluorescence) as a result of the activation of the Oct-4 promoter **(B, right),** that was very intense in some of the cells and absent in MDSC

from the WT mouse used as control (not shown). The location of this expression was cytoplasmic because the reporter gene product remains there rather than translocate to the nuclei as Oct-4a. Some small gfp+ cells were also seen loosely attached to other types of cells in the pP2-pP5 cultures suggesting that they associate transiently with the other pre-plating fractions, and may be found there too. Second, the alkaline phosphatase reaction, a feature of embryonic stem cells, was in general moderately positive in all three cell types, with more intense staining in some individual cells in the Mst KO and mdx MDSC **(bottom)**. However, partial osteogenic differentiation activating alkaline phosphatase cannot be excluded.

The expression of other stem cell related genes in the three types of MDSC was studied using DNA microarray analysis of a panel of 260 stem cell-related genes. **Table 1** shows that there are not substantial differences in the expression of most well known embryonic stem cell genes such as c-Myc, Oct-4 **(Pou5),** alkaline phosphatase 2 and 5, telomerase reverse transcriptase, leukemia inhibitory factor **(LIF),** and mastermind like 1, among the many other related genes. This agrees with the fact that the multilineage differentiation capacity of these MDSC seems to be qualitatively similar among the three types, as shown by the generation in neurogenic medium of cells expressing the neuronal marker NF70 **(Fig. 2),** and in fibrogenic medium of cells, presumably myofibroblasts, expressing α-smooth muscle actin **(ASMA).** However, the proportion of positive cells was lower in mdx and Mst KO MDSC and the cells expressing NF-70 lacked the more apparent neuronal morphology of the differentiated WT MDSC**.** The three MDSC cultures also differentiated into cells expressing calponin as smooth muscle cell marker and von Willebrand factor as endothelial cell marker (not shown).

The lack of active myostatin or dystrophin inhibits the ability of MDSC to undergo in vitro skeletal myogenesis, and this is associated with down-regulation of some critical genes

The WT MDSC forms large polynucleated myotubes expressing MHC II in confluent cultures upon incubation for 1 to 2 weeks in the Hedrick's myogenic medium for adipose tissue

derived stem cells **(Fig. 3 A)**. However, remarkably neither the mdx MDSC nor the Mst KO MDSC **(B)** were able to generate any myotube under these conditions, even after 4 weeks. This is also illustrated in the western blot analysis where the strong MHC II 210 kDa band in the WT MDSC extract is not seen in the confluent Mst KO or mdx MDSC. Immunofluorescence for MHC II detected its high expression in the robust myotubes from WT MDSC **(D)**, but again no MHC II or myotubes were found in the Mst KO or mdx MDSC confluent cultures.

Confluent WT MDSC formed myotubes not just in Hedrick's medium, where Mst KO (not shown) or mdx MDSC did not **(Fig. 4 A),** but also in medium with 10% or 20% FBS **(B),** although myotubes were larger and multinucleated in Hedrick's medium**.** This indicates that cell to cell contact is sufficient to trigger MDSC myogenesis, rather than requiring growth factor depletion. No adipogenesis was detected with Oil red O in Hedrick's medium (not shown). The strong myogenic ability of WT MDSC was confirmed by western blots of parallel confluent cultures, where MHC-II was expressed in all media (triplicate cultures), although more intensively in Hedrick's **(C).** Remarkably, there was no difference in MyoD expression among the different media**.**

The inability of confluent Mst KO or mdx MDSC in several media to form myotubes was irrespective of passage. Myotube formation by WT MDSC cultures persisted for up to 40 passages, although the size and number of the myotubes started to decline as the passage number increased. Cultures of pP5 or pP5 from Mst KO or mdx mice obtained during the pre-plating procedure also failed to generate skeletal myotubes. Despite the drastic obliteration of MHC II+ myotube formation in confluent Mst MDSC and mdx MDSC, the transcriptional expression of myogenesis related genes in the respective proliferating cells was, as in the case of the stem cell genes in Table 1, very similar. For instance, expression of BMPRs (bone morphogenic protein receptors), the Wnt signaling receptors frizzled and jag, IGF1, Notch 1, and Notch 3, was not reduced in these MDSC as compared with the WT MDSC **(Table 2).** However, six notable differences were noticed in which each gene was substantially down-

regulated to a similar extent in the Mst KO and mdx MDSC, versus a strong expression in the WT MDSC. They are Spp1 (secreted phosphoprotein 1, or osteopontin), Actc 1 (cardiac α-actin), MyoD1, cadherin 15, Myf 5, and Notch 2 (see discussion). In contrast, other cadherins (11 and 6), related to neuromuscular development, were up-regulated by 9 and 4-fold, respectively, in both the Mst KO and mdx MDSC. Other than these, there was a virtual 98% similarity among the three MDSC types in terms of the 260 genes investigated.

These results were corroborated by RT/PCR for some of the mRNAs described on the tables. **Fig. 5 A** shows the gel electrophoretic pattern after staining with ethidium bromide, and **B** presents the densitometric values of each band from triplicate determinations corrected by the housekeeping gene values**.** These ratios are comparable among the three MDSC cultures for each gene, but not among the different genes for each culture, because of the different number of cycles applied for the respective transcript amplification. Actc1, Acta1, and MyoD are significantly down-regulated in Mst KO and mdx MDSC as compared with WT MDSC, and Pax 3 is overexpressed, in good agreement with the DNA microarrays. Interestingly, the Oct-4 MDSC resemble the WT MDSC in terms of transcriptional profiles except for Spp1, myoD, and Bmp 4 (not shown).

The WT MDSC are refractory to in vitro modulation of myogenic differentiation by a series of potential modulators, which are also unable to turn on myogenesis in Mst KO and mdx MDSC

Incubation of WT MDSC for 3 days with 5-azacytidine, a demethylating agent and potent inducer of myogenic capacity in pluripotent cell lines **(11,14)** prior to their reaching confluency and switching to myogenic medium, did not stimulate myotube formation. This procedure failed completely to induce it in Mst KO and mdx MDSC (not shown). This suggests that MDSC may turn out to be very refractory to the usual modulators of myogenesis. The first ones tested with a similar paradigm were myostatin, which would be expected to down-regulate myotube formation

in the WT MDSC, and follistatin, that should exert the opposite effect by binding myostatin. **Fig. 6 A-D** shows that the area occupied by MHC II + myotubes was not reduced in the cultures treated with 2 μg/ml myostatin **(B)**, or increased by 0.5 μg/ml follistatin **(C),** as compared to untreated controls **(A)**. Changes were not significant **(D).** Follistatin did not induce any myotube formation by Mst KO and mdx MDSC (not shown). This failure of myostatin and follistatin to affect myogenesis in any type of MDSC occurred despite these cells express the myostatin receptor ActRIIb, in the three cultures, as shown by western blot **(F)**, implying that they should be responsive to exogenous myostatin. Endogenous myostatin expression was not detected in any untreated culture (not shown), even if TGFβ1, another key member of the TGFβ family was expressed **(E).** Finally, neither the monoclonal nor the polyclonal antibodies against myostatin affected myogenesis in the WT MDSC or induced it in the Mst KO or mdx MDSC, as compared to the respective cultures incubated with control IgG (not shown).

Transfection of the MDSC with the AdV Mst cDNA construct, or alternatively with the AdV Mst shRNA which also expresses beta galactosidase did not affect myogenesis in WT or induced it in Mst KO and mdx MDSC. This, despite myostatin and beta galactosidase were respectively expressed. Since we had shown previously **(47)** that 100 nM testosterone or 25 nM DHT stimulated myogenesis in the multipotent cell line C3H 10T(1/2), confluent WT MDSC in Hedrick's medium from the three sources were incubated in triplicate with or without the male sex steroids. No stimulation of MHC-II expression or myotube formation was found by immunocytochemistry and western blot, despite the latter detected a robust expression of the androgen receptor 118 kDa protein, albeit only in WT MDSC. The unresponsiveness of the confluent WT MDSC exposed for 2 weeks to potential modulators of myogenesis replenished fresh with each change of Hedrick's medium, was extended to include TGFβ1 (5 ng/ml), thyroid hormone (TH/T3) (2.4 ng/ml), SNAP as NO donor (5 uM), and SNAP and PDE5 inhibitor tadalafil (20 nM). None of them induced myogenesis in Mst KO or mdx MDSC.

WT MDSC stimulate myofiber regeneration and reduce fat infiltration in the injured gastrocnemius of the aged mdx mice, and Mst KO MDSC recover this capacity in vivo

The failure of the mdx MDSC to form myotubes in vitro in any of the studied conditions was further supported by a comparison of their relative ability to incorporate nuclei into myofibers in vivo. DAPI-labeled cells were implanted into the cryo-lacerated gastrocnemius of the aged mdx mouse and frozen tissue was examined by MHC II immunofluorescence after 2 weeks. **Fig. 7 A** shows that the blue fluorescent WT MDSC nuclei are detected in many of the red fluorescent myofibers and many of these nuclei are central, as may be expected from regenerating myofibers (yellow arrows). Other nuclei are seen in the interspersed connective tissue among the fibers. In contrast, the mdx MDSC nuclei remain interstitial, and no intra-myofiber DAPI-labeled nuclei were seen in a series of adjacent sections **(B)**. The Mst KO MDSC were not tested in this model. The WT MDSC significantly stimulated the appearance of central nuclei on hematoxylin/eosin stained frozen tissue sections in the notexin-injured mdx muscle of aged animals in comparison to control injured muscle receiving vehicle **(D).** Surprisingly, the Mst KO MDSC, that failed to undergo myogenesis in vitro, were able to increase significantly the number of central nuclei in the myofibers **(C, D).** However, this stimulation of myofiber repair did not surpass the efficacy of the WT MDSC **(D),** in contrary to what was originally expected from the absence of myostatin in the Mst KO MDSC.

These results were supported by the fact that both WT MDSC and Mst KO MDSC significantly increased the expression of MCH-II in the notexin-injured mdx aged muscle estimated by western blot, as compared to the vehicle-injected muscle, albeit in this case Mst KO MDSC was slightly more effective than WT MDSC **(Fig. 8).** It should be emphasized that this measurement was conducted in the central region of the muscle, distant from the notexin-injured sites at both ends of the muscle used for the tissue section studies, suggesting that the stimulatory effect on MHC-II expression by MDSC may have been even higher in the injured tissue.

Both WT and Mst KO skeletal muscles are dystrophin +, as seen on frozen sections by the sarcolemma immunofluorescence around the myofibers **(Fig. 9 A),** and in both cases some residual undifferentiated/non-fused dystrophin + MDSC can be seen 3 weeks after implantation into the dystrophin – injured muscle of aged mdx mice, as groups of interstitial mononucleated MDSC dispersed among some of the fields **(B)**. However, only a few myofibers in the MDSC-implanted mdx muscle show partial dystrophin + staining of the sarcolemma, suggesting that the MDSC myofiber differentiation or fusion is very modest.

As expected, fat infiltration is visible in the injured gastrocnemius from vehicle-injected aged mdx mice, mainly interstitially but also as Oil Red O + small regions around or inside myofibers **(Fig. 10 A, B).** WT MDSC were effective in reducing significantly this fat infiltration, and Mst KO MDSC also induced a decrease, although it was not significant **(C).**

DISCUSSION

To our knowledge this is the first report testing the myogenic capacity of MDSC isolated from transgenic mice with inactivation of either the myostatin or the dystrophin genes, in comparison to the wild type MDSC, both in vitro and in the injured muscle of the aged mdx mice in vivo **(28,43)**. Our main findings were: a) in contrast to WT MDSC, Mst KO and mdx MDSC are unable to form myotubes in vitro, or, in the case of mdx MDSC, incorporate their nuclei into the myofibers of injured skeletal muscle in aged mdx mice; this occurs despite no major differences were found among the three MDSC cultures in terms of morphology, replication rates, expression of most members of a subset of key embryonic-like stem cell and other markers, and multi-lineage differentiation other than skeletal myogenic conversion; b) a fundamental difference is however, that the expression of key genes in myogenesis seen in WT MDSC such as actc1, acta1, and myoD, is virtually obliterated in Mst KO and mdx MDSC; c) the three types of MDSC are refractory in vitro to modulation or induction of myotube formation by

well known regulators of this process or of myofiber number, such as myostatin inhibition or overexpression, follistatin, androgens, nitric oxide, and others, despite some of the respective receptors are expressed in MDSC cultures; d) the myogenic, anti-fat deposition and repair capacity of WT MDSC is evident even in the environment of a severely injured mdx muscle at an age where lipofibrotic degeneration is considerable; e) these capacities, blocked in cell culture conditions, are recovered in Mst KO MDSC when these cells are implanted in the injured mdx aged muscle setting, even if not at the level expected from the absence of myostatin.

The WT MDSC used here as control, fulfill all the criteria that have been extensively defined as potential tools for skeletal muscle, cardiac, and osteogenic repair upon implantation into the target organs **(33,38).** In the current work, MDSC were isolated as the pP6 fraction using the preplating procedure on collagen-coated flasks, and shown to have the expected morphology, rapid replication for at least 50 passages, express Sca1, and the ability differentiate in vitro into multiple cell lineages. The latter capability includes a robust formation of multinucleated and branched myotubes, that is assumed to translate in vivo into their ability to donate their nuclei to injured skeletal myofibers or at least to paracrinely stimulate their regeneration. This is evidenced by a much higher number of centrally located nuclei, and even some central location of the DAPI-labeled implanted nuclei. In previous studies we had shown that WT MDSC generate at least smooth muscle and epithelial cells when implanted into urogenital tissues **(29,30)**, adding up to the extensive demonstration of their stem cell nature **(7,12,28,50).** Another novel finding here is that WT MDSC have some embryonic-like stem cell features, mainly the expression of Oct-4, myc, LIF, and other embryonic stem cell genes. These MDSC contain both tiny rounded cells similar to the very small embryonic-like stem cells **(VSEL)** described in many adult organs **(49)**, and other larger ones, that in the case of the myostatin + and dystrophin+ Oct-4 MDSC are identified by the activation of the Oct-4 promoter denoted by expression of gfp as reporter protein.

Another finding is the unexpected observation that myotube formation by the WT MDSC in vitro is refractory to modulation by agents that are well known to affect this process, or skeletal muscle mass in vivo. The fact that myotube formation by WT MDSC was not influenced by: a) downregulation or overexpression of myostatin, despite the detectable expression of its receptor (ActIIb); b) counteracting myostatin activity by the respective antibodies or follistatin, that in vivo stimulate myofiber growth **(18-20);** c) incubating with androgens that induce myofiber growth **(21-23),** or nitric oxide that stimulates satellite cell fusion in vitro **(24,25)**, poses questions related to the role of MDSC during normal myogenesis. A study showing that myostatin stimulated fibroblast proliferation in vitro and induced its differentiation into myofibroblasts, while increasing TGFβ1 expression in C2C12 myoblasts, did not examine MDSC differentiation **(12).** The claim of a small inhibitory effect of myostatin on the fusion index in MDSC **(50)** may indicate less fusion efficiency but might not entirely reflect the actual effects on the number and size of myotubes, as determined here. This question requires further clarification in terms of the actual modulation of MDSC differentiation.

It may be speculated that satellite cells rather than MDSC are the only myogenic progenitors during normal myofiber growth, as opposed to repair of damaged fibers **(51)**. Therefore the selected in vitro conditions may not mimic the repair process, or alternatively unknown in vivo paracrine or juxtacrine modulators may modify the response of MDSC to the better characterized agents tested in this work. Another possibility is that myostatin and other modulators investigated here would stimulate in vivo satellite cell replication and fusion to the adjacent myofibers to induce hypertrophy, without truly affecting MDSC differentiation or fusion.

We are unaware of any report on the isolation or characterization of MDSC from the Mst KO or the mdx mice. Therefore, it is also both novel and unexpected to find that these cells obtained from the same skeletal muscles, using identical procedures, and displaying rather similar non-myogenic pluripotency and stem cell marker features, are however completely unable to form myotubes in vitro or, in the case of the mdx MDSC to fuse with myofibers in vivo.

In fact, our prediction was that both types of cells should be more myogenic than the WT MDSC, in the case of Mst KO MDSC because of the absence of myostatin, and in the case of mdx MDSC because they originate from muscles with a considerable regeneration ability occurring after the spontaneous bouts of necrosis that characterize this strain**.** In this context, bone marrow stem cells isolated from the mdx mouse are also unable to undergo myogenesis, despite their wild type counterparts are **(52)**

The fact that Mst replenishment, either as recombinant protein or as cDNA does not counteract the unexpected myogenic inhibition found in the Mst KO MDSC, suggests that these MDSC are not actively involved in the skeletal muscle hypertrophy that characterizes the Mst KO mouse **(45).** The inability to undergo myogenesis is not due to myostatin depletion per se, but rather to unknown effects of this gene inactivation, presumably on other myogenic pathways that may be defective. Although in the case of mdx MDSC we did not perform a gain of function experiment with dystrophin or its DNA, we may speculate that, considering the high regenerating ability of mdx satellite cells **(53),** it is likely that this gene inactivation that leads to necrosis does not however impair myogenesis in vivo, and that the mdx MDSC would not participate in this process. This conjecture is also supported by the fact that, unlike the WT MDSC, the mdx MDSC implanted into the injured gastrocnemius of the mdx mouse did not fuse with preexisting myofibers or originate centrally located nuclei. However, validation of both assumptions requires further tests with other approaches. We cannot speculate whether it is the absence of dystrophin or the down regulation of its down-stream pathways during gestation that inhibits myogenesis.

Perhaps the most interesting result is the activation of the in vitro-suppressed myogenesis in Mst KO MDSC, and/or the ability to fuse with preexisting myofibers, after their implantation into the notexin-injured mdx gastrocnemius. At the age selected (10 month-old), this muscle experiences the considerable damage that occurs in the diaphragm much earlier **(3,4),** and this is compounded by injury. It may be speculated that the restoration of Mst KO

MDSC in this setting occurs by paracrine or juxtacrine modulation, possibly of some of the key genes silenced in these cells. Estimation of their products and proof-of-function approaches may elucidate this issue. The fact that despite Mst KO MDSC being able to fuse with or differentiate into new myfibers, they do not increase the muscle repair process in a clearly more efficient way than WT MDSC, may possibly result from the persistent myostatin expression in the fibers that may counteract its absence in Mst KO MDSC. This suggests the need to block systemically myostatin in the host muscle, not just in the implanted MDSC.

The reason why neither Wt KO MDSC nor mdx MDSC can undergo myotube formation in vitro may lie on “imprinted” genetic modifications on the expression of certain key genes for skeletal muscle differentiation caused by the absence of myostatin and dystrophin expression, respectively, that so far have not been characterized. One such a gene is cardiac α-actin (Actc) the major striated actin in fetal skeletal muscle and in adult cardiomyocytes, but strongly down-regulated in adult skeletal muscle to 5% of the total striated actin **(54)**, whose mRNA is highly expressed in the proliferating (non-differentiating) WT MDSC but at very low level in the Mst KO and mdx MDSC. The same applies to the α1-actin (Acta1) mRNA, the adult protein encoding thin filaments **(55).** Since actins are so crucial for cell division, motility, cytoskeleton, and contraction, and mutations are associated with severe myopathies, it would not be surprising that their down-regulation could cause to the lack of myogenic commitment in Mst KO and mdx MDSC.

Similarly, the striking transcriptional down-regulation of myoD, a critical early gene in skeletal myogenesis **(56)**, and of secreted phosphoprotein 1, or osteopontin, a gene mostly involved in ossification, inflammation, and fibrosis, but postulated recently to participate in early myogenesis and skeletal muscle regeneration **(57)**, may also trigger the absence of myogenic capacity in Mst KO and mdx MDSC. Interestingly, the fact that Pax 3 mRNA, upstream of MyoD in the myogenic signaling **(58)** is expressed at the same levels in Mst KO and mdx MDSC and higher than in WT MDSC, suggests that the myogenic commitment of Mst KO and mdx MDSC

is arrested at some point in between these genes. Since a critical regulator of skeletal muscle development, Mef2a (Myocyte enhancer factor 2a) **(59)**, is expressed similarly in the three MDSC (as Pax 3 is), albeit at very low levels, the silencing may occur at the level of the satellite cell marker, Pax 7. Therefore, it is not surprising that expression of a member of the cadherin family (cadherin-15) that is involved in later stages such as myoblast differentiation and fusion **(60)** is obliterated in these MDSC.

In conclusion, our results show that MDSC obtained from wild type and transgenic mice lacking either myostatin or dystrophin express Oct-4 and other embryonic like stem cell genes and appear similar in most features, except for the null or poor expression in Mst KO and mdx MDSC of some critical early genes. These genes encode factors critical for myogenesis and for maintaining the integrity of myotubes and myofibers, thus possibly leading to their inability to form myotubes in vitro or donate their nuclei in vivo. This would imply in the case of mdx muscle that satellite cells, that are key players in the bouts of muscle repair following necrosis, are not affected, but it is unknown to what extent the inhibition of MDSC myogenesis programmed by the absence of dystrophin may affect this repair. In turn, the genetic inactivation of myostatin induces similar, albeit counterintuitive, effects, and it is counteracted by cross-talk with myofibers and other cell types in the host mdx muscle. Although our results do not prove conclusively the initial working hypothesis that myostatin inactivation would enhance in vitro and in vivo the myogenic capacity of MDSC, this possibility still needs further in vivo testing by blocking myostatin not just in the implanted MDSC but in the host muscle with follistatin, shRNA, antibodies, or other procedures. Finally, systemic muscle-targeted WT MDSC implantation appears as a promising approach worth exploring to stimulate repair and avoid fat infiltration in the aged dystrophic muscle, or even in local administration for limited muscle injury.

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LEGENDS TO FIGURES

Figure 1. Key embryonic stem cell markers are expressed in MDSC irrespective of whether myostatin or dystrophin is genetically inactivated. A: representative pictures of proliferating MDSC that were subjected to immunocytochemistry for Oct-4, showing nuclear location in most cells (200X). B: left: homogenates from the same cell cultures were subjected to western blot for Oct-4 (nuclear: 45 kDa; cytoplasmic:33 kDa); right: the pP6 cell fraction from the muscle of the Oct-4 Pr-gfp mouse (Oct-4 MDSC) shows cytoplasmic green fluorescence for gfp (200X). C: proliferating MDSC were subjected to cytochemistry for alkaline phosphatase (200 X).

Figure 2. Myostatin or dystrophin genetic inactivation does not block the multipotent non-myogenic differentiation capacity of MDSC. Representative pictures of proliferating MDSC treated for 2 weeks in differentiation media and subjected to immuno-cytochemistry for NF-70 (A) and ASMA (B) to detect marker expression of neural cells and myofibroblasts (200 X).

Figure 3. Myostatin or dystrophin genetic inactivation blocks the myogenic differentiation capacity of MDSC. A, B: representative pictures of confluent MDSC from the three mouse strains maintained for 2 weeks in myogenic medium ("Hedrick's") and subjected to immunocytochemistry for MHC II to detect differentiation into polynucleated myotubes (magnifications as indicated). C, left: western blot for MHC II (210 kDa); C, right: blue/red merge of confluent MDSC in myogenic medium labeled with DAPI and submitted to immunofluorescent detection of MHC (200X).

Figure 4. The potent myotube forming capacity of WT MDSC in myogenic medium is decreased but still maintained under high serum concentrations, in the presence of steady MyoD expression. A: representative micrographs of myotubes generated in confluent WT MDSC maintained for 2 weeks in Hedrick's medium (left), and of confluent mdx MDSC that

fail to originate myotubes (right), as evidenced by immunocytochemistry for MHC II (200 X). B: similar WT MDSC cultures in PM with 20% or 10% serum (200X). C. triplicate western blots for WT MDSC in the above cultures in the three types of media subjected to immunodetection for MHC II (210 kDa) and MyoD (44 kDa). 10: 10% PM; 20: 20% PM; H: Hedrick's medium

Figure 5. Confirmation by RT/PCR of selected differences in transcriptional expression in the three types of undifferentiated MDSC, previously detected by DNA microarrays. RNAs obtained from triplicate cultures of proliferating MDSC, consecutive to those used for DNA microarrays on Tables 1 and 2, were subjected to RT/PCR with specific primers spanning an intron for the number of PCR cycles stated in parenthesis, as follows: Actc1 (30), Acta1 (30), MyoD1 (33), Pax3 (28), and GAPDH (26). A: ethidium bromide-stained agarose gels; B: densitometry of relative band intensities referred to housekeeping gene for the indicated number of PCR cycles. Controls without reverse transcriptase were blank. *: $p < 0.05$ **: $p < 0.01$; ***: $p < 0.001$.

Figure 6. Myostatin and follistatin fail to modulate the myogenic differentiation of MDSC, despite myostatin receptor is expressed. A-D: confluent WT MDSC in myogenic medium were incubated in triplicate on 6-well plates for 1 week with recombinant myostatin (B) or follistatin (C) or with no addition (A), subjected to immunocytochemistry for MHC II (40X). The relative area occupied by the MHC II + myotubes was estimated by quantitative image analysis (15 fields/well/3 wells) (D). Cont: control; Mst: myostatin; Fst: follistatin. No myotubes were formed in confluent Mst KO and mdx MDSC under any treatment (not shown). E-F: western blot detection in confluent MDSC from the three mice strains of the expression of the ActRIIb (E) and TGFβ1 (F). Myostatin was not detected.

Figure 7. Implanted WT MDSC stimulate myofiber repair in the injured gastocnemius of the old mdx mouse, while Mst KO MDSC act similarly to WT MDSC and mdx MDSC fail to trigger this process. A,B: Aged (10-month old) mdx mice were used to maximize myofiber loss and lipofibrotic degeneration in the gastrocnemius. Muscles wer cryoinjured and implanted with

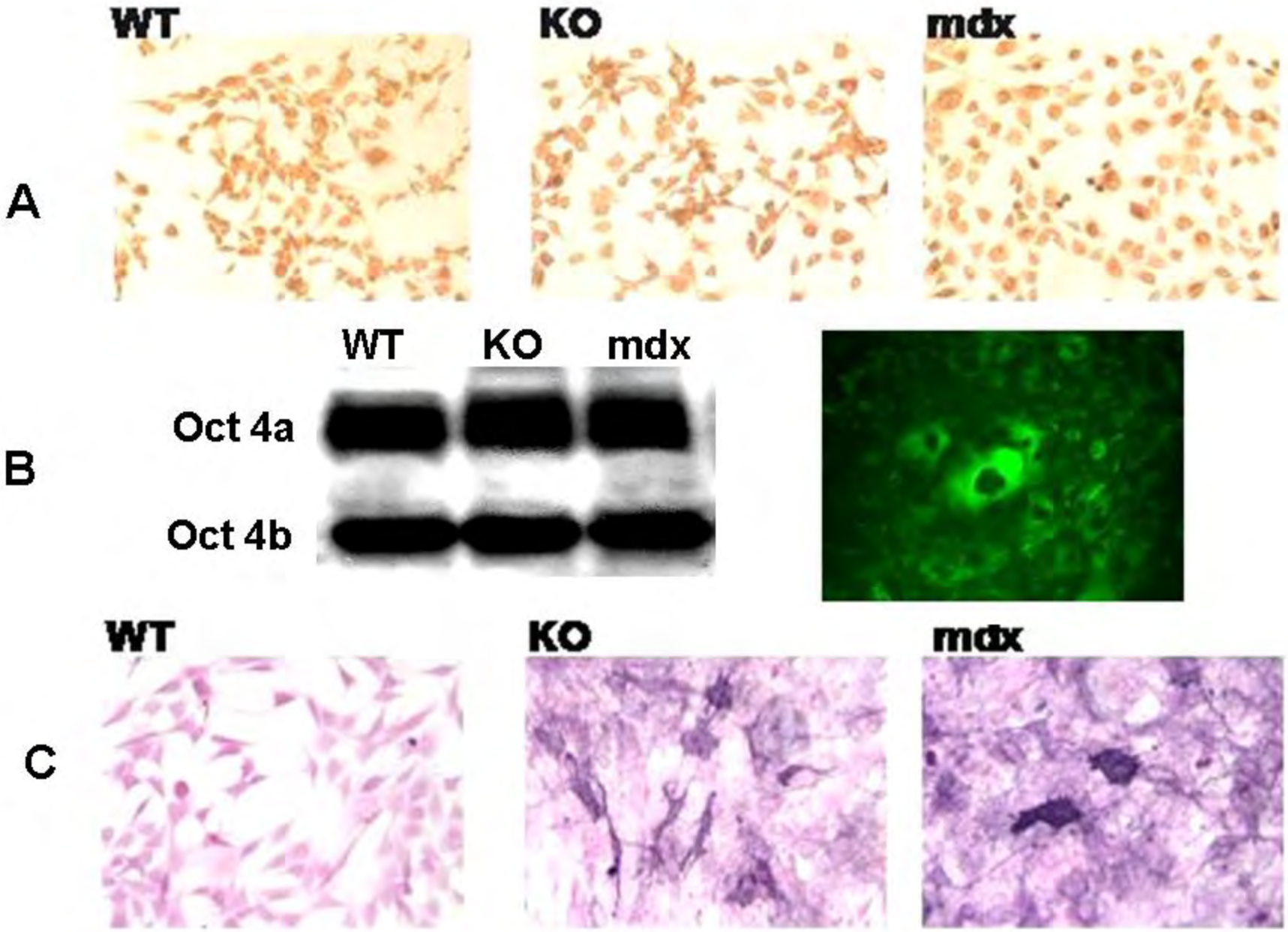
0.5×10^6 DAPI-labeled WT MDSC (A) or mdx MDSC (B), and allowed to undergo repair for 10 days. Frozen muscle sections were stained for MHC-II with Texas red streptavidin, and the merge of blue and red fluorescence was obtained (200X). MDSC nuclei centrally located within myofibers are indicated with yellow arrows. C: Gastrocnemius injury in the aged mdx mice was performed in the two apexes of the muscle with notexin, and muscles were injected 4 days later with saline or with 1.0×10^6 WT MDSC or Mst KO MDSC in saline (n=5/group). Repair was allowed to proceed for 3 weeks. Hematoxylin eosin staining was performed in frozen sections and a representative picture shows myofibers from the gastrocnemius implanted with WT MDSC with arrows pointing to abundant central nuclei (200X). D: quantitative image analysis of these tissue sections (WT), in comparison to tissue sections from Mst KO MDSC-implanted mice (KO) and saline-injected controls, based on 12 fields per section, 3 sections per animal. *: $p < 0.05$

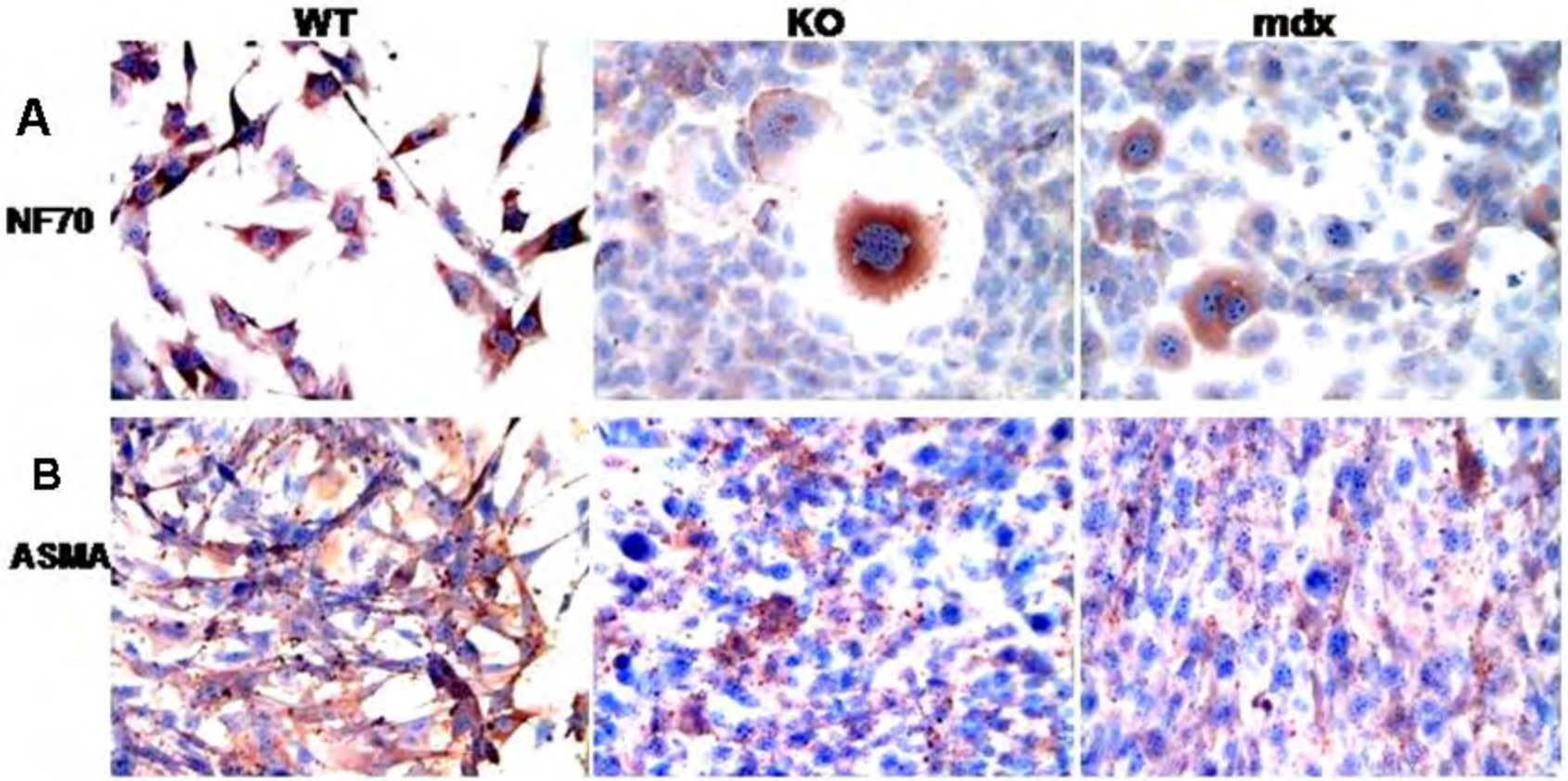
Figure 8. WT and Mst KO MDSC stimulate MHC-II expression in the injured mdx mouse gastrocnemius. Top. Western blot analysis for MHC II and GAPDH (reference gene) in muscle tissue homogenates prepared from the central region of the muscles that were examined histochemically in Figure 7 C. Each lane corresponds to an individual mouse homogenate (n=5/group), and the three gels were run simultaneously. Bottom. Densitometric evaluation of the relative intensity expressed as ratios of the MCH-II and GAPDH bands. *: $p < 0.05$

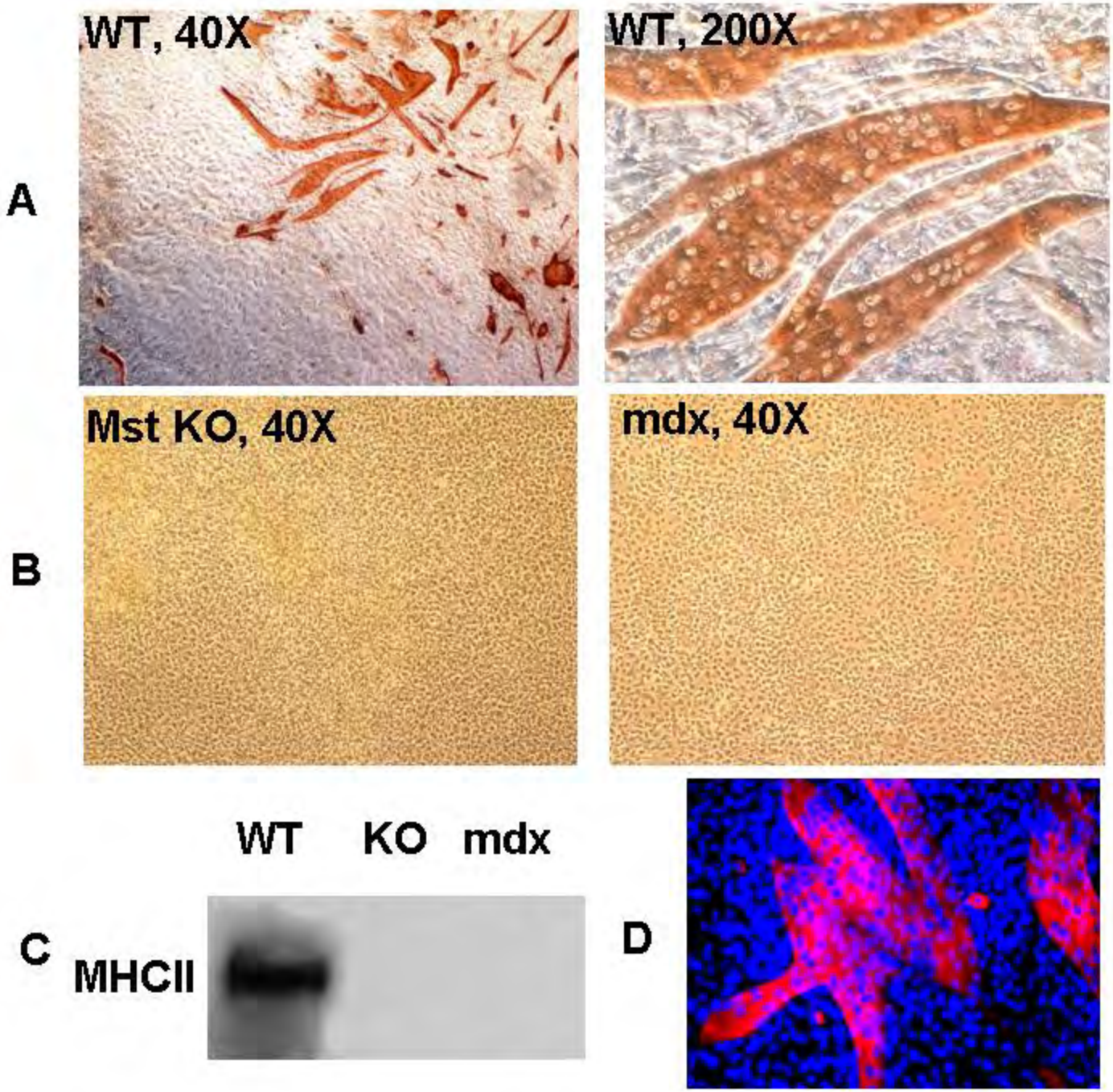
Figure 9. Dystrophin + MDSC restore some dystrophin expression in the injured mdx gastrocnemius, but only in sparse areas. A. Myofibers from the intact gastrocnemius from the WT mouse, the source of WT MDSC, show positive immunofluorescence for dystrophin (nuclei stained with DAPI) (200X). B: WT MDSC implanted in the injured mdx gastrocnemius are still identified after 3 weeks as groups of mononucleated dystrophin+ cells in some tissue sections (200X). C: in other tissue sections, MDSC appear to have fused with the mdx myofibers that show irregular dystrophin + staining; however, most of the sections were

negative for dystrophin. D: the same field as in C examined under visible light confirming the integrity of the myofibers including the dystrophin – area.

Figure 10. WT MDSC reduce fat deposit in the injured mdx mouse gastrocnemius. A,B: Representative picture of a positive field from frozen tissue sections from the untreated mdx injured gastrocnemius, adjacent to the ones shown on Fig. 8 C, fixed in formalin and stained with Oil Red O, showing mostly interstitial fat and occasional myofiber fat infiltration (200X). Staining in the sections from the muscle implanted with WT and Mst KO MDSC was similar, but in sparser locations. C: quantitative image analysis of the tissue sections from the three rat groups, based on 12 fields per tissue section and the total positive area per section (%), calculated as a mean for 3 adjacent sections per rat, and 5 mdx mice/group. *: $p < 0.05$


WT
KO
mdx
A
WT
KO
mdx
Oct 4a
Oct 4b
B
WT
KO
mdx
C


WT
KO
mdx
A
NF70
B
ASMA


A
WT, 40X
WT, 200X
B
Mst KO, 40X
mdx, 40X
C
WT
KO
mdx
MHCII
D

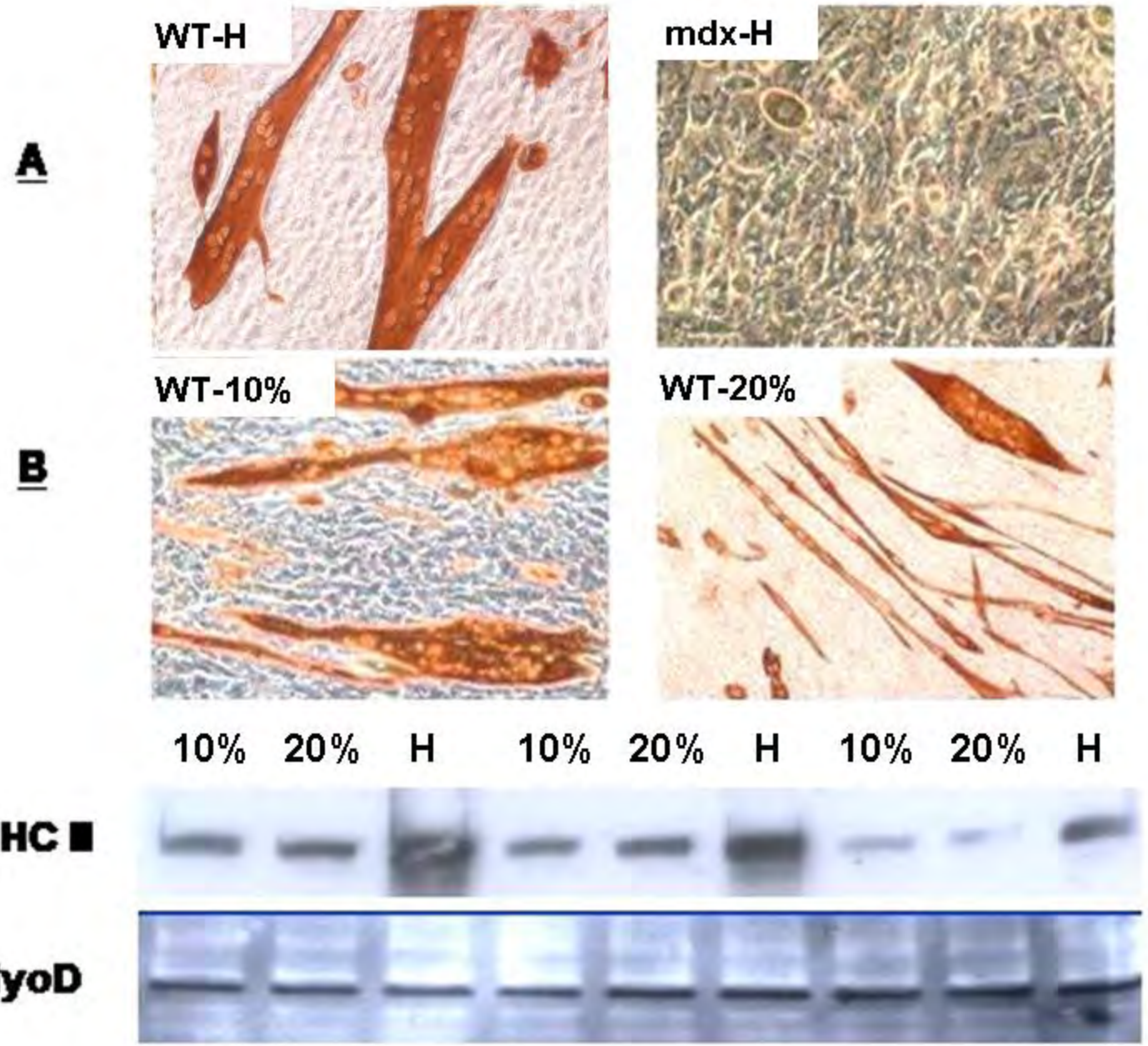

A
WT-H
mdx-H
B
WT-10%
WT-20%
10% 20% H 10% 20% H 10% 20% H
C
MHC
MyoD

TABLE 1
SOME STEM CELL-RELATED GENES ARE TRANSCRIBED SIMILARLY IN MDSC IRRESPECTIVE OF MYOSTATIN OR DYSTROPHIN GENETIC INVACTIVATION

RNA from the three MDSC types at 80% confluence (not undergoing myogenesis) were treated by DNAse and submitted to DNA microarrays. Some key stem cell genes are selected. Values are relative expression levels normalized by housekeeping genes. GAPDH expression was 98-102

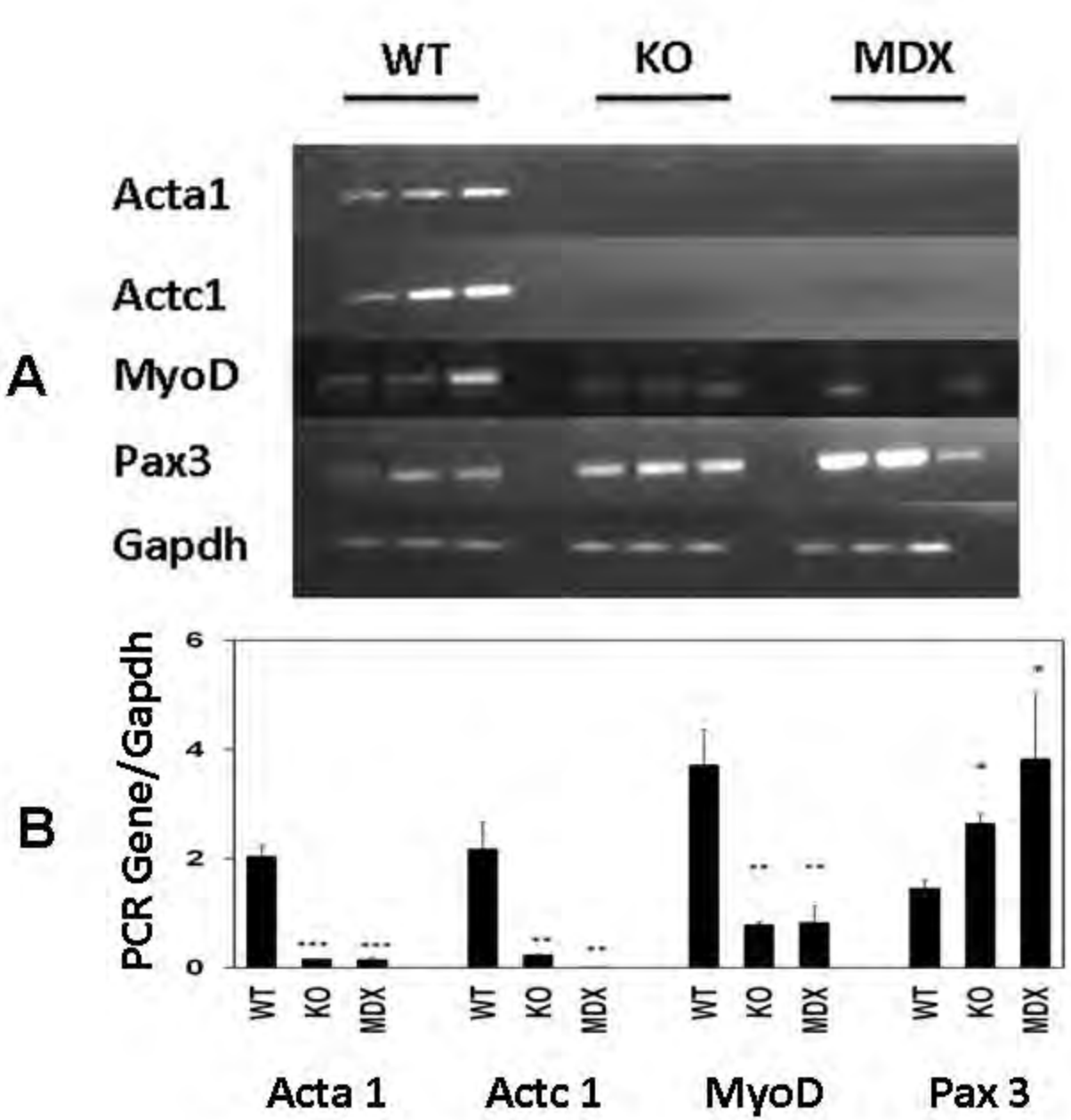
		WT	KO	mdx
Myc	Myelocytomatosis oncogene	12.4	18.1	15.0
Pou 5M	Pou domain (Oct4)	10.1	16.7	11.5
Alp 2	Alkaline phosphatase 2	6.4	6.9	6.3
Alp 5	Alkaline phosphatase 5	1.2	1.6	1.2
Tert	Telomerase reverse transcriptase	1.0	1.0	0.7
Utf 1	Undifferentiated embryonic cell TP1	1.0	0.8	1.0
Man 1	Mastermind like 1	13.1	16.7	12.8
Lif	Leukemia inhibitory factor	1.5	0.9	0.9
PPARγ	Peroxisome proliferating ARγ	1.1	1.8	1.5

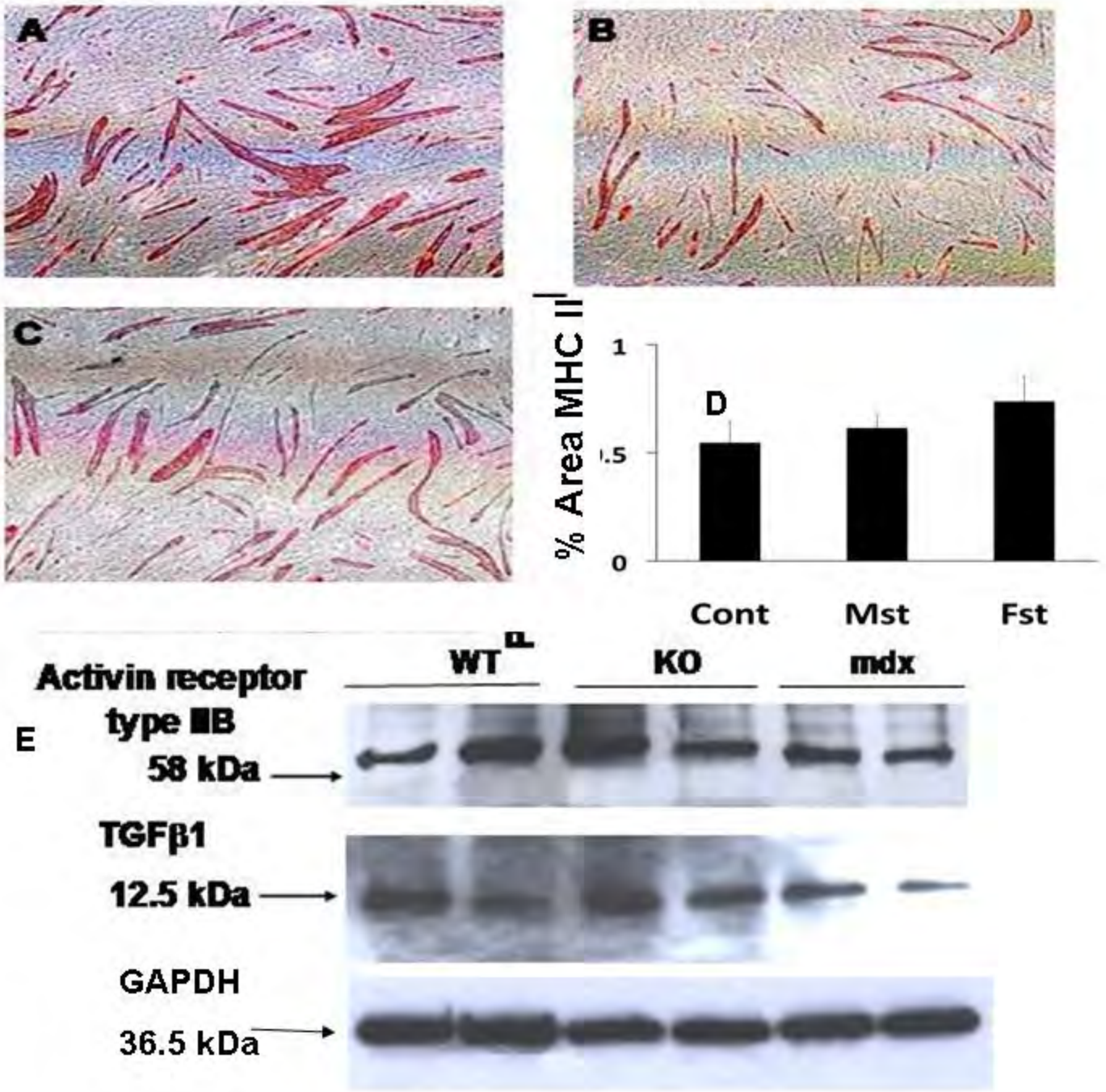
TABLE 2

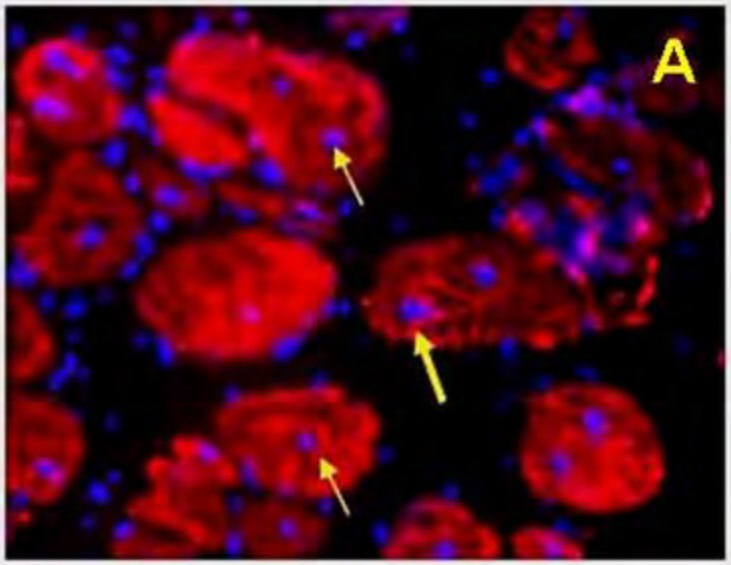
SOME SKELETAL MYOGENESIS-RELATED GENES ARE CONSIDERABLY DOWN-REGULATED IN MDSC BY MYOSTATIN OR DYSTROPHIN GENETIC INACTIVATION, WHEREAS OTHERS REMAIN UNCHANGED.

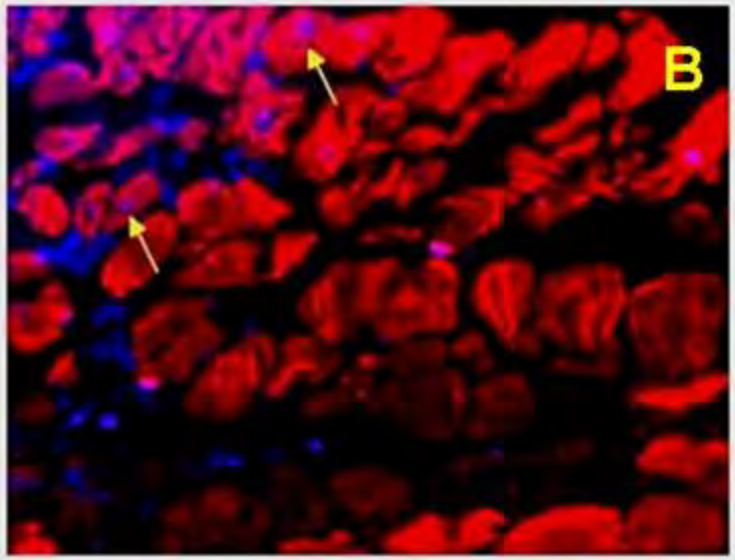
Some key myogenesis related genes are selected. See Table 1 for further description

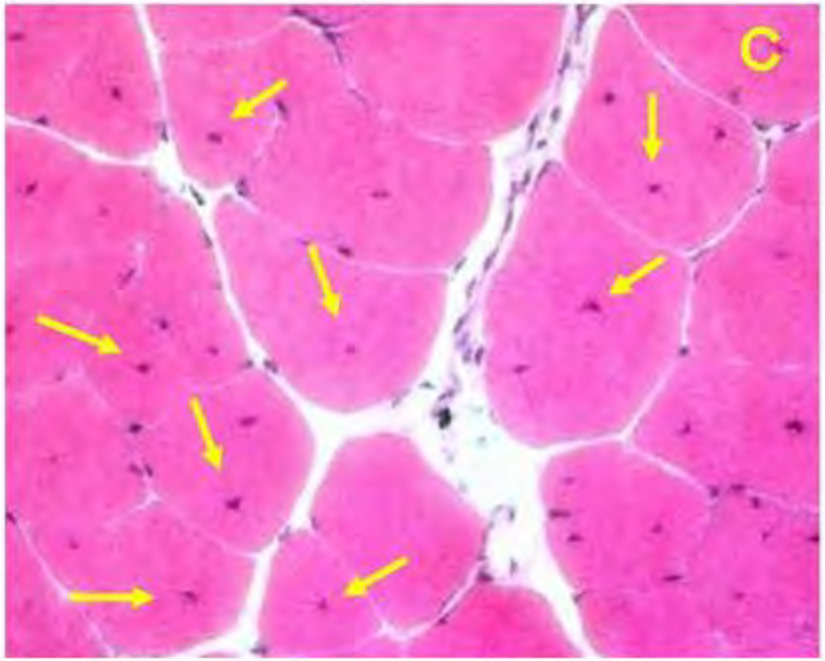
		WT	KO	mdx
SPP 1	Secreted phosphoprotein 1 (osteopontin)	70.8	20.3	7.0
Actc 1	α actin (cardiac)	39.9	6.5	4.9
Myo D1	Myogenic differentiation 1	17.5	2.7	2.7
Cadherin 15	Cadherin 15	8.7	1.7	1.3
Myf 5	Myogenic factor 5	4.2	2.7	2.7
Notch 2	Notch gene homolog 2	4.2	2.8	2.6
BMPR 2	Bone morphogenic receptor 2	23.3	20.3	19.5
BMPR 1a	Bone morphogenic receptor 1a	8.1	10.4	8.3
BMPR 1b	Bone morphogenic receptor 1b	0.8	0.8	1.0
BMPR 4	Bone morphogenic protein 4	2.7	2.7	2.8
IGF 1	Insulin-like growth factor 1	5.1	4.2	5.3
Jag 1	Jagged 1	2.8	3.4	5.1
Fzd 1	Frazzled homolog 1	2.7	2.8	3.4
Notch 1	Notch gene homolog 1	2.6	2.7	1.2
Notch 3	Notch gene homolog 3	2.8	2.6	1.3

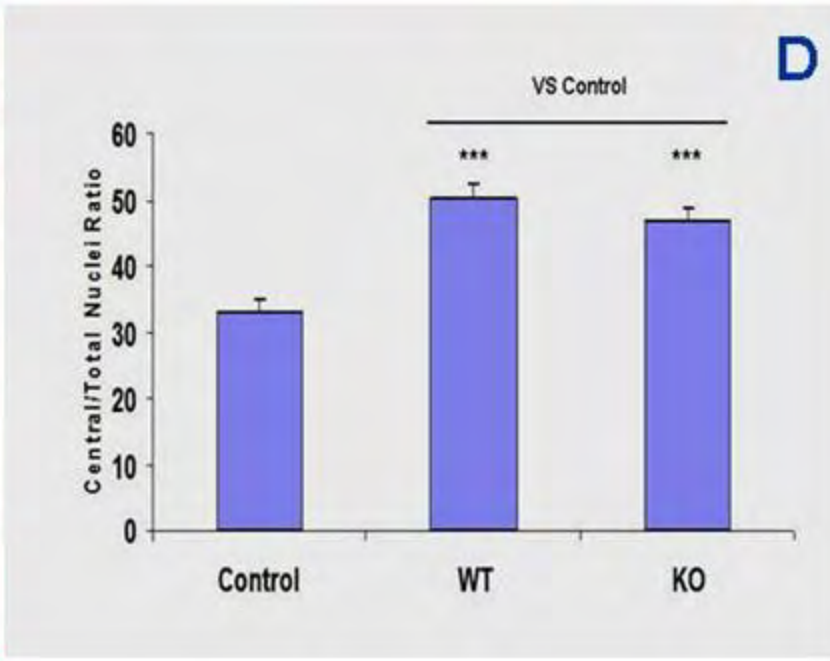

WT
KO
MDX
A
Acta1
Actc1
MyoD
Pax3
Gapdh
B
PCR Gene/Gapdh
0
2
4
6
WT
KO
MDX
Acta 1
Actc 1
MyoD
Pax 3

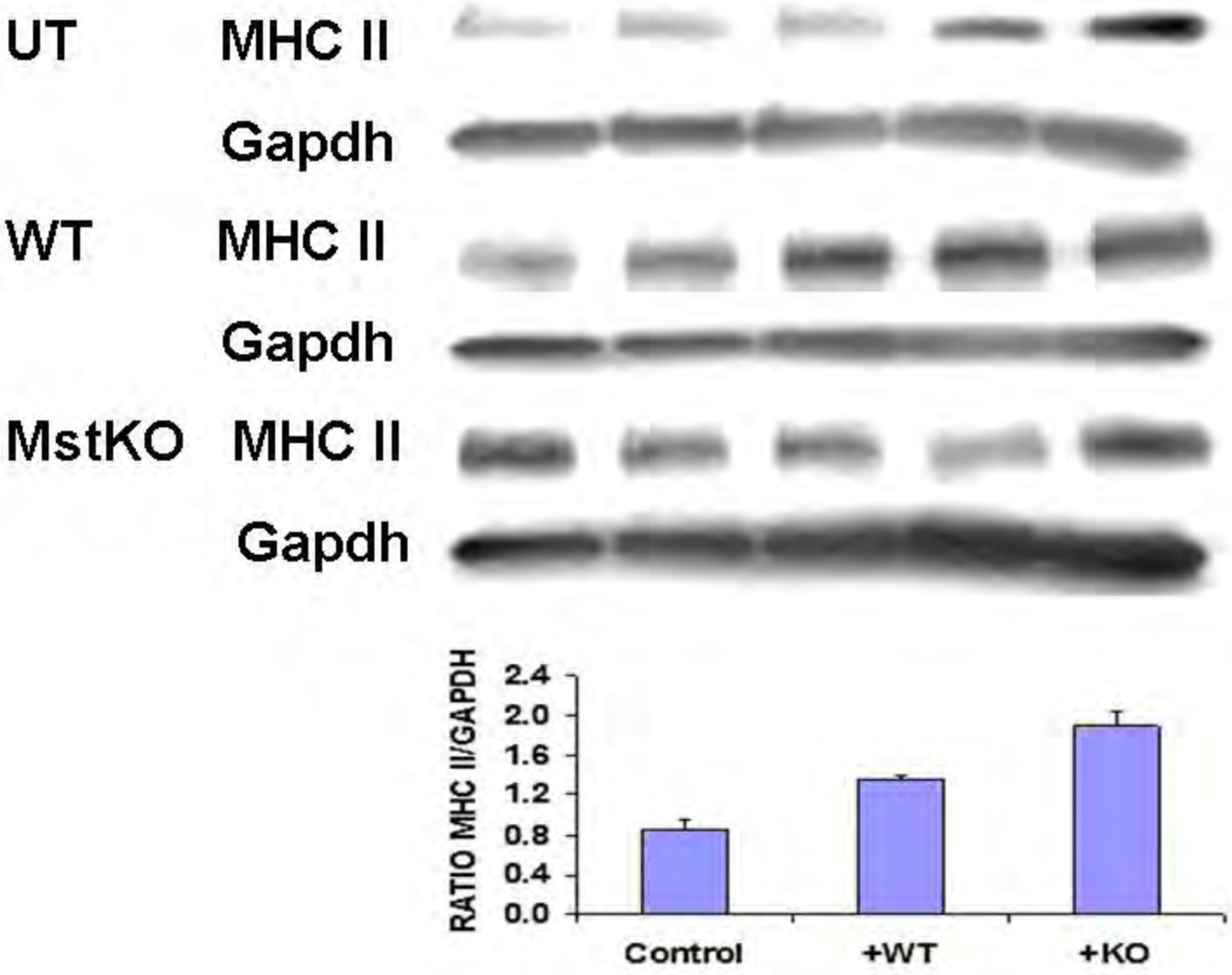

A
B
C
D
% Area MHC II
1
.5
0
Cont
Mst
Fst
E
Activin receptor type IIB
58 kDa
WT
KO
mdx
TGFβ1
12.5 kDa
GAPDH
36.5 kDa

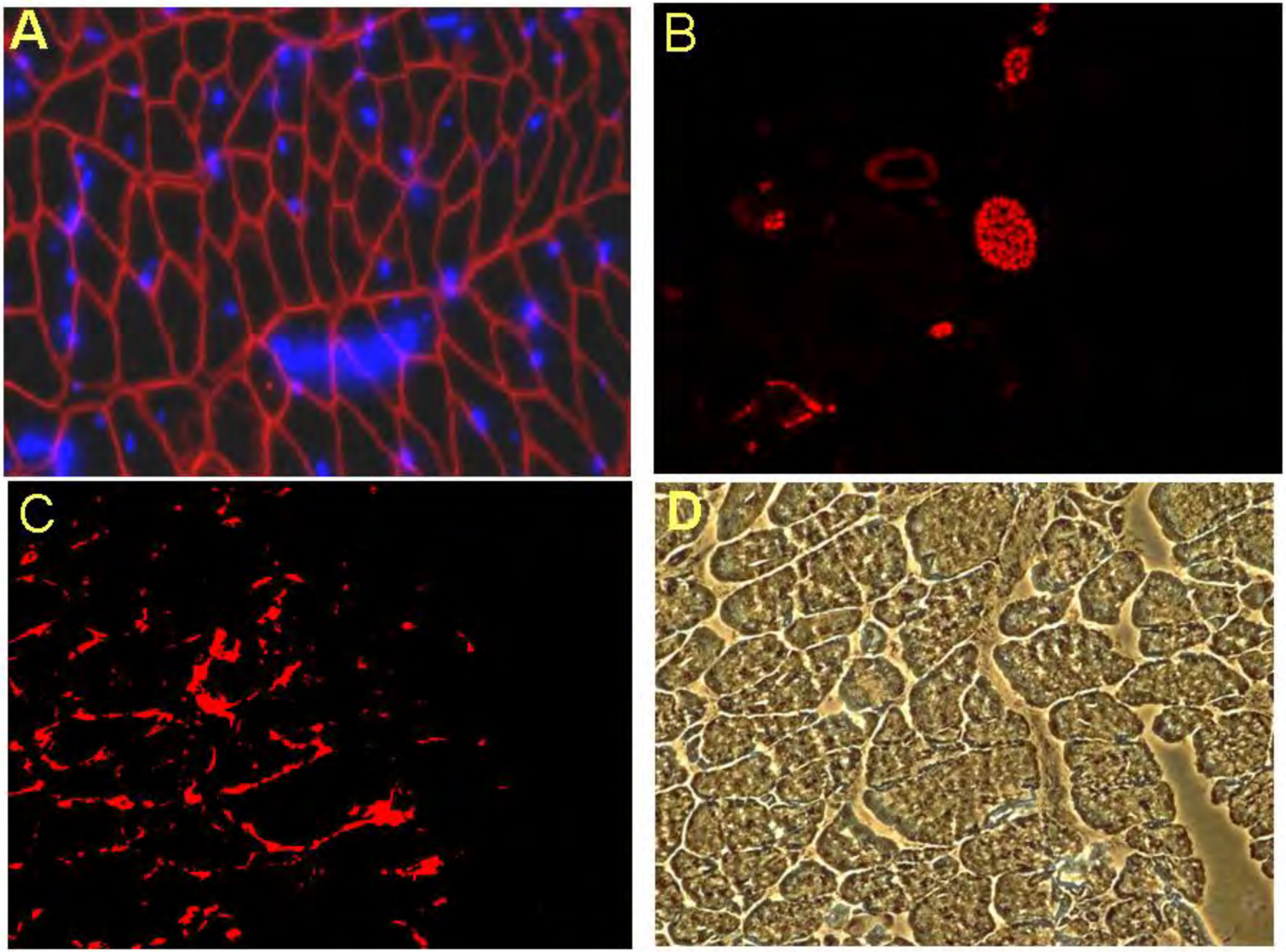

A

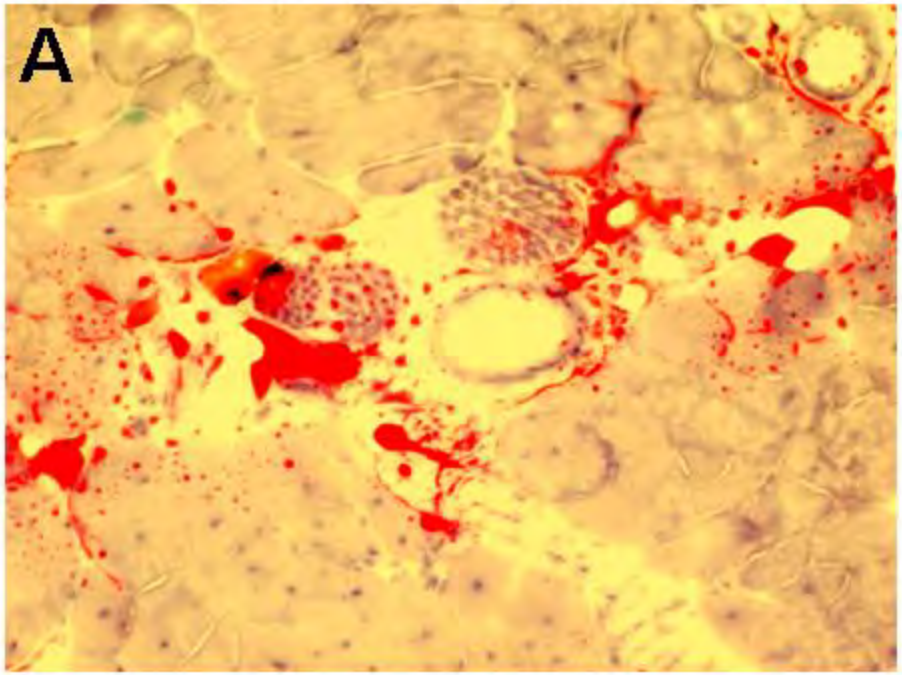

B

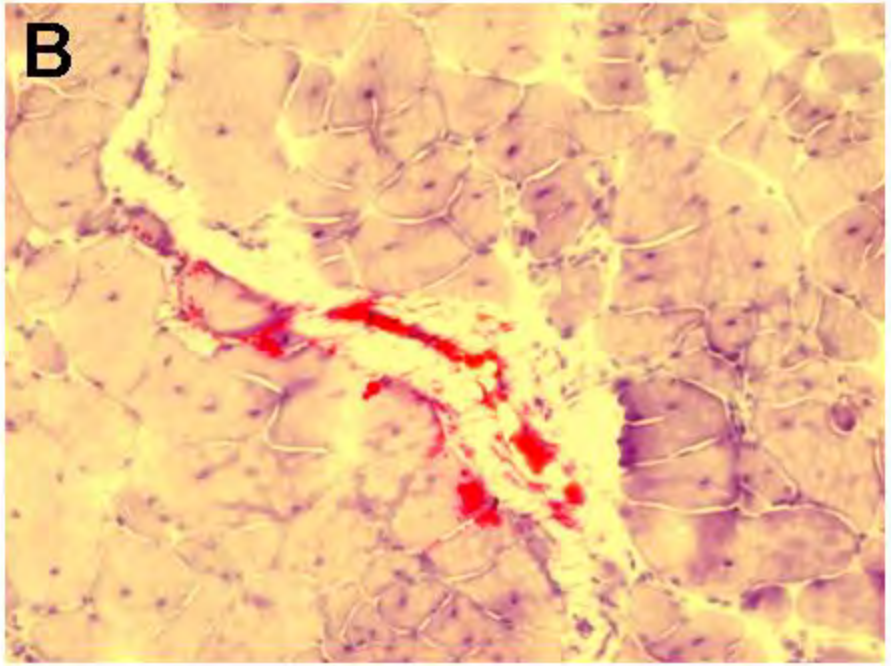

C


D
VS Control
***
***
Central/Total Nuclei Ratio
60
50
40
30
20
10
0
Control
WT
KO


UT
MHC II
Gapdh
WT
MHC II
Gapdh
MstKO
MHC II
Gapdh
RATIO MHC II/GAPDH
2.4
2.0
1.6
1.2
0.8
0.4
0.0
Control
+WT
+KO


A
B
C
D


A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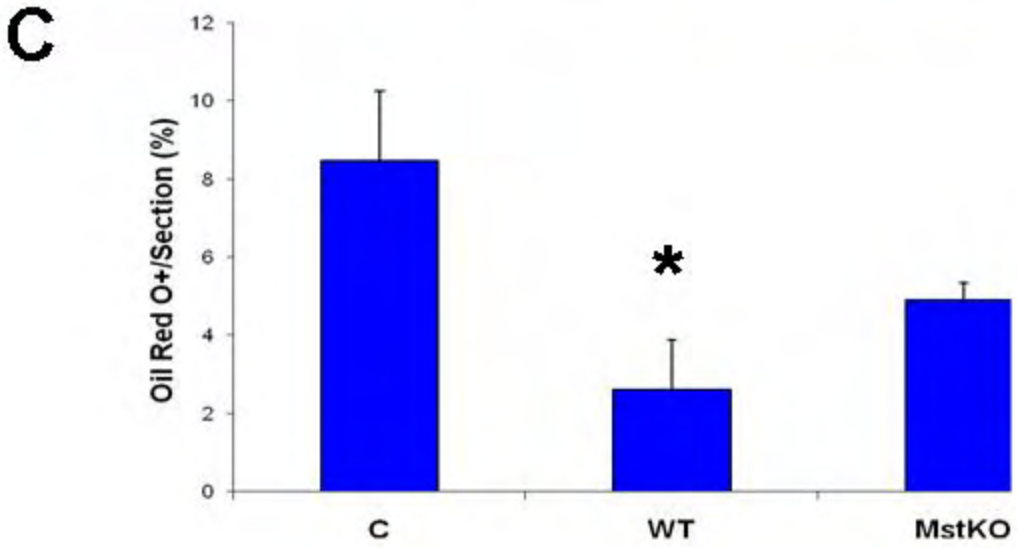
C
Oil Red O+/Section (%)
12
10
8
6
4
2
0
*
C
WT
MstKO